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IV

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THE TOXICOLOGY OF NICKEL CARBONYL.

By H. W. ARMIT.

(*From the Lister Institute of Preventive Medicine.*)

(Four Figures.)

PART I.

Introduction.

Nickel carbonyl was discovered by Mond, Langer, and Quincke (1890). On passing a current of carbon monoxide over finely divided (pyrophoric) metallic nickel, they discovered that a gaseous compound of nickel and carbon monoxide was formed, having the composition of $Ni(CO)_4$.

When the gas is heated to 150° C. it is decomposed into its constituents, and metallic nickel is deposited. The volume of liberated carbon monoxide after dissociation is four times that of the undissociated nickel carbonyl vapour. The gas can be condensed to a mobile liquid, if cooled in a refrigerating mixture. Fluid nickel carbonyl boils at 43° C. and solidifies into needle-shaped crystals at $-25°$ C. The vapour density at 50° C. is 86·9.

Further investigations on this compound were conducted by Mond and Nasini (1891), Berthelot (1901), Mittasch (1902) and Dewar and Jones (1903).

Nickel carbonyl is a clear, pale straw-coloured liquid, volatilising at room temperature. It has a peculiar soot-like smell, which can be detected in extremely small quantities (about 1 volume in 2,000,000), while the Bunsen flame becomes luminous when nickel carbonyl is present in air to the extent of 1 volume in 400,000. The liquid is soluble in alcohol, benzene and chloroform. It is not acted upon by alkalies nor by weak acids. Strong hydrochloric acid does not decompose it, but nitric acid and aqua regia do so easily.

Cobalt does not form a carbonyl, but iron does. Liquid iron carbonyl ($Fe(CO)_5$), when acted on by light, gives off one molecule of carbon monoxide, and a solid diferro-nonacarbonyl is formed, having the composition $Fe_2(CO)_9$. Dewar and Jones (1906) also describe a tetracarbonyl of iron, which occurs in the form of short, dark green, lustrous prisms. It is almost insoluble and is not volatile. The pentacarbonyl is volatile at ordinary temperatures, but not to such an extent as the tetracarbonyl of nickel. It is made by passing carbon monoxide over reduced pyrophoric iron.

The discovery of nickel carbonyl was put to practical use by L. Mond for the manufacture of pure nickel. At first, it was not thought that the compound was more dangerous than the amount of carbon monoxide contained would suggest, and it appears that very little precaution was taken by the early investigators to avoid inhaling the vapour; fortunately without evil results.

However, when the substance was produced on a large scale, the accidental escape of the gas led to some unfortunate accidents, some four of which proved fatal.

The suggestion to investigate the toxicology of nickel carbonyl I owe to Dr Mond, who has placed a supply of nickel carbonyl and iron carbonyl at my disposal, and who has done much to render the work less difficult. Much assistance has been derived from the advice of his chemists, Drs Shields and Hirtz and also from Dr Langer, the Managing Director of the Mond Nickel Company's Works at Clydach, to all of whom I wish to express my thanks. Through the courtesy of Dr Mond I have been able to observe some mild cases of poisoning in man. It is therefore a great pleasure to me to express my thanks to Dr Mond in this place.

I wish also to express my grateful indebtedness to Dr Charles Martin, the Director of the Lister Institute of Preventive Medicine, for the very valuable assistance which he has given me, both in suggesting methods to overcome difficulties and also in criticising results; and to Dr Harden, whose advice on chemical matters has been a great help to me.

The symptoms of nickel carbonyl poisoning in man were as follows: immediately after having been exposed to air containing plant-gas, there was giddiness, and at times dyspnoea and vomiting. These symptoms passed off rapidly, as soon as the patients were brought into the fresh air. After from 12 to 36 hours, the dyspnoea returned, cyanosis appeared and the temperature began to be raised. Cough with more or less blood-stained sputum occurred on the second day or later.

The pulse rate became increased, but not in proportion to the respiratory rate. The heart remained normal. Delirium of varying types frequently occurred, and a variety of other signs of disturbance of the central nervous system was noted. Death took place in the fatal cases between the 4th and 11th days. The chief changes found post mortem were haemorrhages in the lungs, oedema of the lungs, haemorrhages in the white matter of the brain (in one case this was very extensive), while some doubt exists as to whether any blood changes were present.

At present it is not possible to state what the lethal dose for man may be.

In animals poisoned with nickel carbonyl vapour, analogous symptoms and post mortem changes have been observed. It is proposed to deal with these in detail in a subsequent communication. The post mortem changes in animals (rabbits, cats and dogs) consist of haemorrhages in the upper air passages, haemorrhages, oedema and compensatory emphysema of the lungs, haemorrhages in the adrenals and in the central nervous system. Haemorrhages occur less commonly in the kidneys, still less commonly in the spleen and very rarely in the liver.

The inhalation of air containing 0·018 volume $^0/_0$ of nickel carbonyl vapour for 65 minutes is just sufficient to kill rabbits; 0·04 volume $^0/_0$ has to be inhaled for 90 minutes by cats to produce death, while dogs die if exposed to 0·036 volume $^0/_0$ for 90 minutes. No symptoms appear for the first 12 to 24 hours after the inhalation.

A few observers have studied the poisonous effects of nickel carbonyl. Henriot and Richet (1891) came to the conclusion that nickel carbonyl is taken up by the blood and is slowly split up. They found carbon monoxide haemoglobin. Langlois (1891) believes that nickel carbonyl replaces the oxygen in the blood. Vahlen (1891) discusses the conclusions of these authors and comes to the conclusion that death is due to nickel carbonyl as such and not to carbon monoxide. McKendrick and Snodgrass (1891) ascribe the toxic action to nickel, on the basis of a small number of experiments, chiefly dealing with enormously excessive doses. A number of earlier workers have dealt with the toxicology of nickel salts, among whom, Anderson Stuart (1884), Ross (1887), Riche (1888), Laborde and Riche (1888) and Rohde (1889) may be mentioned.

The Toxic Agent in Nickel Carbonyl poisoning.

In considering what the toxic agent in nickel carbonyl poisoning may be, one has to deal with three possibilities:

1. That the carbon monoxide of the compound is wholly or partly responsible for the symptoms;
2. that nickel carbonyl is absorbed as such and acts toxically;
3. that the nickel of the compound produces the symptoms.

A modification of these may be considered, viz. that nickel carbonyl is absorbed as such and that the nickel being set free later, acts toxically in the tissues.

1. *Consideration of what part in the Toxicology may be played by Carbon Monoxide.*

When nickel carbonyl is dissociated, the carbon monoxide liberated occupies four times the volume of the original vapour. Shields has pointed out that if nickel carbonyl is shaken up with blood, the haemoglobin acquires the colour characteristics of carbon monoxide haemoglobin. In my poisoning experiments, when the minimum doses of vapour were used, the blood contained only a small amount of carbon monoxide, which seldom exceeded 5 %.

In order to show that carbon monoxide is not the toxic agent, the following facts can be brought forward:

1. In fatal poisoning from minimal doses the actual quantity employed is insufficient when combined with the haemoglobin to produce more than 5 % of saturation with carbon monoxide, and this amount is harmless.
2. The blood of an animal saturated to the extent of 32 % with carbon monoxide loses carbon monoxide during subsequent poisoning by nickel carbonyl.
3. Iron carbonyl is less toxic than nickel carbonyl, although it contains more carbon monoxide.

Ad. 1. It has been found in a large number of experiments, that the inhalation of 0·018 volume % of nickel carbonyl vapour in air for 65 minutes is sufficient to kill rabbits; 0·04 volume % for 90 minutes is sufficient to kill cats, and 0·036 volume % for 90 minutes to kill dogs. It cannot be supposed that 0·072 volume %, 0·16 volume % and 0·144 volume % of carbon monoxide in air when inhaled by rabbits, cats and dogs for 65, 90 and 90 minutes respectively would produce fatal results. These figures represent the amount of carbon monoxide liberated, if all the nickel carbonyl were dissociated. According to Gruber (1883) 0·07 to 0·08 volume % of carbon monoxide even when inspired for days does not kill rabbits. Gréhant (quoted by Sachs, 1900) found that dogs die when exposed to 0·4 to 0·5 volume % for from ¾ to 1¾ hours.

During fatal poisoning in rabbits, samples of blood were taken and examined spectroscopically and by Haldane's carmine method (1895) for carbon monoxide haemoglobin. If carbon monoxide had been the toxic agent, one would have expected to find at least 50 % of the haemoglobin saturated with carbon monoxide at the end of the inhalation period.

Ad. 2. Rabbits were exposed to carbon monoxide gas until the haemoglobin of the blood showed from 25 to 32 % saturation. 30 % of the haemoglobin of the first rabbit was saturated with carbon monoxide; the rabbit was then allowed to breathe air containing 0·0188 volume % of nickel carbonyl for 65 minutes, and died without exhibiting any additional symptoms. 25 % of the haemoglobin of the second rabbit was saturated with carbon monoxide, and this rabbit was exposed to the same dose of nickel carbonyl. It too lived as long as the control and did not show any additional symptoms. 28·5 % of the haemoglobin of the third rabbit was saturated with carbon monoxide and then the rabbit was exposed to 0·019 volume % of nickel carbonyl for 65 minutes. At the end of the inhalation the carbon monoxide haemoglobin had decreased to 25·7 % of the total. Two other animals after inhaling sufficient carbon monoxide to saturate 25 and 32 % of the haemoglobin of their blood respectively, recovered after severe illness on being poisoned by a dose of nickel carbonyl which killed 83·1 % of rabbits subjected to it.

The toxic effect of nickel carbonyl is therefore not increased by a preliminary inhalation of carbon monoxide, provided that not more than 32 % of the haemoglobin is saturated. The amount of carbon monoxide combined with the haemoglobin may actually diminish during the inhalation of nickel carbonyl.

Ad. 3. Nickel carbonyl contains less carbon monoxide than iron carbonyl. As has been stated, 0·018 volume % inhaled for 65 minutes of nickel carbonyl vapour in air is sufficient to kill rabbits. 0·025 volume % of iron carbonyl vapour is required to be inhaled for one hour to produce the same effects.

It will be shown subsequently that the course of the symptoms and the death in nickel carbonyl poisoning is not like that of carbon monoxide poisoning.

Conclusion.

The foregoing facts prove that the toxic effect of nickel carbonyl is not produced by its carbon monoxide content. They also show that when the quantity of the nickel carbonyl vapour inhaled is only just sufficient to kill, the total available amount of carbon monoxide is insufficient to influence the course of poisoning.

2. *Consideration of what part in the Toxicology may be played by Nickel Carbonyl as such.*

In order to attack the problem on safe grounds, it was found necessary to ascertain how nickel carbonyl behaves when it comes into contact with the fluids and tissues of the body. This study involved the investigation of the following:

(1) Under what conditions nickel carbonyl is dissociated.

(2) What the solubility of nickel carbonyl in water at various temperatures and pressures is.

(3) What the solubility in blood and in serum is.

(4) Whether any compound of nickel carbonyl or nickel is formed in the blood, and

(5) What becomes of the nickel in the body after any dissociation which may take place, has occurred.

Vapour Tension of Nickel Carbonyl.

The vapour tension of nickel carbonyl at temperatures between – 9° and + 39° C. was determined by Mond and Nasini (1891), Dewar and Jones (1903) and Mittasch (1902). The curves of these observers showed moderate agreement. It was, however, deemed advisable to redetermine the vapour pressure of the particular samples used in this investigation. The following method was employed. A tube was cleaned and closed at one end and then carefully dried. It was then filled with dry mercury and boiled out. The tube inverted over a bath of mercury was jacketed with water and a thermometer was also introduced into the jacket. A small tube provided with two stopcocks and with the lower end curved upwards for a few millimetres was then completely filled with nickel carbonyl, care being taken to exclude air in the lower portion. The lower curved end was then inserted under the open end of the barometer tube and the lower tap opened. The nickel carbonyl was driven out of the small apparatus by warming the tube with the hand. The temperature was rapidly lowered to about 3° C. and then slowly taken up to 35° C. and again depressed to about 5° C. The vapour pressure at different temperatures was as follows:

Up values		Down values	
2·85° C.	167·61 mm.	34·25° C.	547·8 mm.
7·1	194·61	26·3	410·97
11·8	230·56	22·45	349·97
18·9	300·5	15·35	273·51
26·4	403·47	9·9	218·66
34·85	564·3	6·4	190·66

Fig. 1 is the curve interpolated from these values. The points determined by Dewar and Jones are given for comparison. The values determined at the lower and higher temperatures practically coincide, while the values in the middle of the curve (between 19° and 24° C.) are slightly lower than those determined by Dewar and Jones. The curve interpolated from these observers' determinations would therefore lie slightly higher and would be flatter.

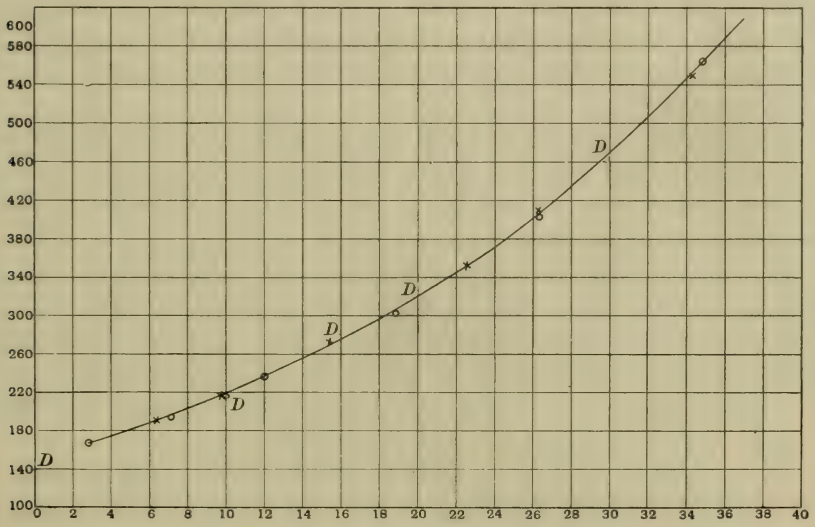

Fig. 1.

O=up values. X=down values. D=Dewar and Jones's points.

Dissociation.

When nickel carbonyl is kept in the presence of air and moisture, a deposit forms. This is due to the dissociation of the compound and the formation of a hydrate or carbonate of nickel.

In the burette containing nickel carbonyl, a deposit of hydrate or carbonate separates out after a few hours if any air is present. It is extremely difficult to keep liquid nickel carbonyl clear on account of this spontaneous dissociation, and the only safe way to avoid any deposit of hydrate is to fill the tube or burette with carbon monoxide after careful cleaning and drying.

Dewar (1903) noticed that when dissociation takes place out of contact with air and in the presence of an excess of carbon monoxide, recombination of part of the nickel with carbon monoxide occurs on cooling. In the vapour tension experiments, visible dissociation (*i.e.* black deposit on the tube) was seen at and above 22° C. Reversible action was noticed when a tube containing nickel carbonyl vapour and sealed at both ends, was heated at one spot. Under these circumstances a mirror of nickel is deposited on the glass, and at the periphery of the mirror a slight blackening is seen. This is most intense close to the edge of the mirror and diminishes imperceptibly further away from it. On cooling, some or all of this black deposit or even some of the mirror disappears again.

Some tubes of nickel carbonyl vapour with and without hydrogen were prepared and the dissociation watched. Evidence of reversible action was seen both in the tubes of pure nickel carbonyl and in those containing nickel carbonyl and hydrogen.

Slight dissociation was seen when tubes of nickel carbonyl vapour were kept in the water bath at 18° C. for several days. Dissociation takes place though slowly at lower temperatures. At room temperature (about 16° C.) a slight amount of black deposit was seen after 20 days in a flask containing water saturated with nickel carbonyl free from oxygen. At 35° C. nickel carbonyl dissociated fairly rapidly.

In an experiment dealing with the solubility of nickel carbonyl in water (to be described later) about 1·4 c.cm. of liquid nickel carbonyl was allowed to volatilise completely in an exhausted litre flask containing water at 35° C. Within 10 minutes a heavy black deposit of metallic nickel had separated out, and this increased steadily during the following two hours. The vapour pressure increased by 3 mm. in 10 minutes, and at the end of 20½ hours it had increased by 31 mm. from the carbon monoxide set free. In a similar experiment, carried out at 23·4° C., enough nickel carbonyl was introduced into the flask to give a vapour pressure of 250 mm. After 20 minutes, the total pressure had increased by 11 mm.

A flask containing water, some mercury and an excess of nickel carbonyl, prepared anaerobically, was kept in the water bath at 29·7° C. After 14 days a black deposit had formed, in the fluid a thick layer was seen on the surface of the mercury and some black precipitate was adherent to the glass flask and capillary tubes.

When nickel carbonyl is evaporated into a large space containing air, a smokiness is seen, which may be due to floating particles of nickel hydrate or carbonate in extremely fine division.

Solubility of Nickel Carbonyl in Water.

The small solubility of nickel carbonyl and the fact that it dissociates so readily and also is apt to form an emulsion render it extremely difficult to determine the actual amount of nickel carbonyl taken up by water and aqueous fluids. The chief difficulties are due, firstly, to the fact that when nickel carbonyl in liquid condition is in excess, an emulsion is formed and the liquid does not settle readily, so that samples of the solution drawn off for examination frequently contain small amounts of liquid nickel carbonyl in suspension, and, secondly, that in dealing with partial pressures, dissociation takes place sufficiently extensively at temperatures above 10° C. to alter the pressure before equilibrium between the gases and the liquid has been obtained.

Solubility at Constant Temperatures and Varying Pressures.

The experiments were conducted at 9·8° C. The observations were made upon solutions which had been shaken with excess of liquid nickel carbonyl or had been saturated with gas at different partial pressures.

METHODS.

1. *Experiments with Liquid Nickel Carbonyl in excess.*

A small flask similar to those employed for washing gases, but with tubes of capillary bore, and provided with stopcocks, was filled with boiled water, after having been washed out with hydrogen. The water was boiled in a large flask and carried over into the smaller flask by its own steam pressure. During the cooling process the tap in connection with the longer tube was left open and connected with a capillary tube filled with mercury and dipping into a mercury bath. After the temperature had fallen to room temperature, the mercury and some water were sucked out through the tap in the short tube into an exhausted flask. Nickel carbonyl was then placed into a burette with two stopcocks, between two seals of mercury, and this burette having been applied to the longer tube (all tubes being filled with mercury) both the tap of this tube and the lower one of the burette were opened, and nickel carbonyl allowed to flow into the flask together with the mercury. The tap was then closed, the flask well shaken and kept at a constant temperature in the water bath. The flask was kept in the water bath for about 24 hours during the earlier experiments, but when it was found that complete solution occurred within one hour, the sojourn was shortened to this period. The flask was shaken at frequent intervals while in the bath.

In order to determine the amount of nickel carbonyl dissolved, a sample of the water was sucked out into a second gas washing bottle, which had been well washed out with hydrogen and evacuated. Before taking this sample, the flask was inverted and allowed to stand so that any nickel carbonyl in suspension might settle. A few c.cm. of water were first drawn off through the longer tube, so that, should any globules of nickel carbonyl be sticking to the inside of the capillary tube, these would first be got rid of. The weight of the water drawn off for examination was determined by weighing the second gas washing bottle before and after taking the sample.

The bottle was placed in connection with a hydrogen apparatus and a combustion tube, having two asbestos plugs. The combustion tube rested on two Ramsay burners, being supported by copper troughs with lids. The tube was thoroughly heated, after which hydrogen was bubbled slowly through the fluid to drive off the dissolved nickel carbonyl. The hydrogen was burnt at the further end of the tube. The heat dissociated the nickel carbonyl and caused a mirror of nickel to form on the combustion tube close to the Ramsay burner, while some of the remaining nickel was deposited in the asbestos plugs.

The flame at the pointed end of the combustion tube indicated if the least trace of nickel carbonyl escaped dissociation. This never took place when the tube was properly heated, provided that the hydrogen was bubbled through the solution slowly at the beginning. Toward the end of this stage, the test employed was to allow the tube to cool, while the hydrogen was turned off, and then on lighting the hydrogen again, one could immediately detect if any nickel carbonyl was still present. The flame test is so delicate (detecting 1 volume in 400,000) that no appreciable error occurred in carrying it out.

After the carbonyl had been completely driven off, the nickel mirror in the tube and the nickel in the asbestos plugs were dissolved in nitric acid. The solution was evaporated to dryness, and the residue treated with hydrochloric acid, again evaporated and dissolved in water. The fluid was made up to a given bulk and the nickel determined by the colorimetric method with dimethylglyoxime, described by A. Harden and myself (1906).

2. *Experiments in which Water was brought into equilibrium with the Gas at different Partial Pressures.*

A litre bolt-head flask with a special short neck was fitted with a rubber cork having five perforations. The perforations transmitted (1) a capillary tube dipping down to below the top level of the water, (2) a capillary tube dipping down to nearly the bottom of the flask, (3) a short tube to connect to the water pump, (4) a short tube to connect to the mercury gauge, and (5) a special burette for nickel carbonyl, with a three-way tap, through the one limb of which hydrogen could be let in.

The flask having been exhausted, the water was boiled (the mercury gauge being clamped off during this period to prevent distillation), any air given off was washed out by repeated displacement with hydrogen, and finally the flask was again exhausted. A measured quantity of nickel carbonyl was run into the flask from the burette and the flask was well shaken. A long tube containing mercury, which had been freed from water and air, was then attached to the capillary tube dipping to below the top level of the water. The sample was taken from the tube dipping nearly to the bottom of the flask and as it was being removed, mercury was let in, so that the upper level of the water was kept constant. The sample was dealt with as before.

One or two points in connection with the methods require especial attention. The hydrogen must be free from arsenic. Before displacing the nickel carbonyl, a blank test should be made each time to ensure that the rubber tubes connecting the parts of the apparatus (which should be as short as possible) have not retained any nickel carbonyl from a previous experiment dissolved in the rubber.

Results.

The following table gives the results obtained at 9·8° C. with *varying pressures* of nickel carbonyl :

V. P. in mm.	Mgrs. Ni per 100 grs. H_2O		Calculated for 214 mm.*
214*	—	6·43 6·52 6·35	6·43
186·5	5·53		6·34
138	3·92		6·09
183	5·64		6·59
109	3·32		6·32

* Vapour tension of nickel carbonyl at 9·8° C.

The amount dissolved is directly proportional to the pressure, and these values plotted against the pressures as in Fig. 2 give a straight line.

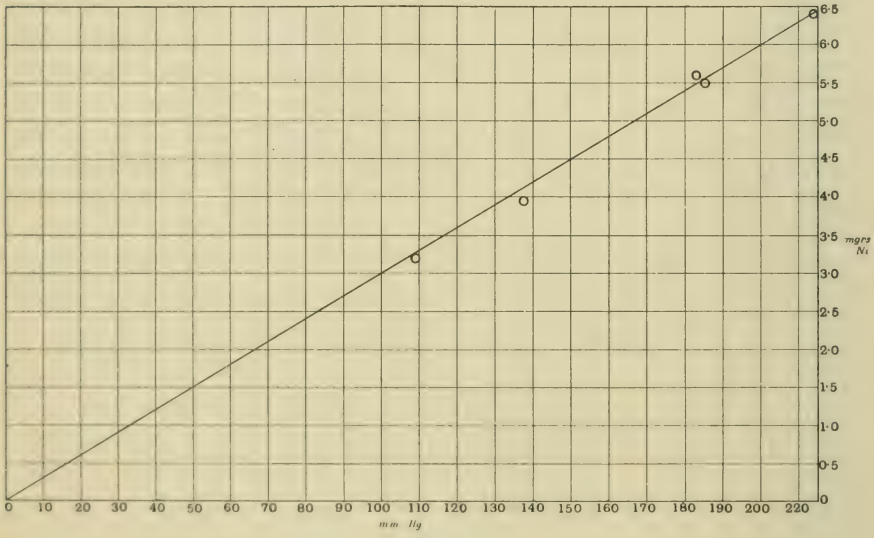

Fig. 2.

Solubility of Nickel Carbonyl at Different Temperatures.

For the determination of the solubility at varying temperatures, the method of saturating water with liquid nickel carbonyl and subsequently withdrawing a sample for analysis had to be employed for all temperatures above 10° C., owing to the quite appreciable dissociation of the gas which takes place leading to too high readings of the partial pressure. The great disadvantage of employing excess of liquid carbonyl is the extreme precaution which has to be taken to avoid any minute particles of nickel carbonyl being carried over into the sampling flask.

The following results were obtained by the two methods employed:

1. Water shaken up with nickel carbonyl in excess.

Temperature	Mgrs. Ni per 100 grs.		Mgrs. $Ni(CO)_4$	$Ni(CO)_4$ in c.cms.
9·8	6·42 6·52 6·35	6·43	18·01	2·36
22·3	4·77 4·81 4·5	4·69	13·63	1·79
29·1	4·41 3·61 3·45	3·82	11·1	1·45

2. Water brought into equilibrium with nickel carbonyl at various partial pressures.

Temp.	V. T. of $Ni(CO)_4$ at temp.	Part. P. of gas used	Mgrs. Ni per 100 grs. H_2O	Calculated for V. T. at temp.	
2·6	169	127	6·9	9·18	
9·8	214	109	3·32	6·32	6·33
		138	3·92	6·09	
		183	5·64	6·59	
		186·5	5·53	6·34	
23·4	363	250	2·13*	3·48	
25	386	215·5	1·45*	2·6	
35	564	132	0·38*	1·69	1·50
		199	0·48*	1·42	
		235	0·7*	1·4	

* These results are certainly too low owing to dissociation.

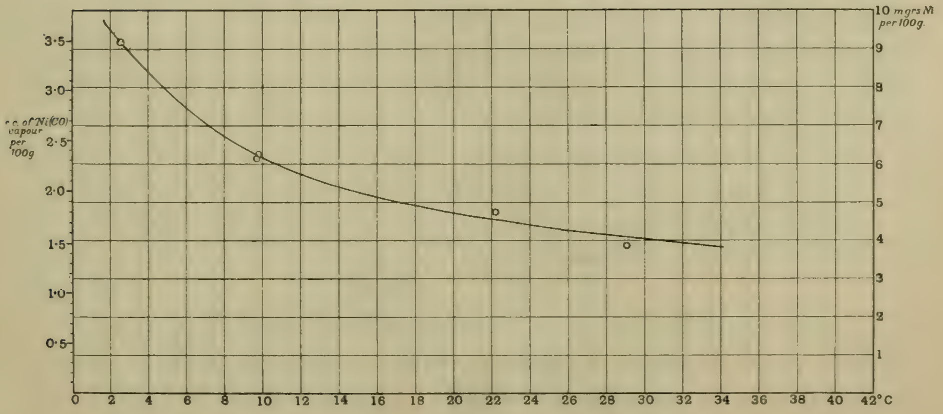

Fig. 3.

Solubility of Nickel Carbonyl in Serum.

The same methods as described above for water were attempted for serum, but it was found that in a viscid fluid the method with excess of liquid nickel carbonyl could not be relied on, since the inclusion of droplets of nickel carbonyl cannot be detected. The second method, therefore had to be resorted to. This, however, yields results which are too low at higher temperatures, on account of dissociation.

In displacing carbonyl from serum, a special gas washing bottle with the inside tube of capillary bore, only reaching to within a few millimetres above the level of the liquid, was used to prevent frothing. The displacement of the gas therefore took much longer than when hydrogen was bubbled through the liquid, and during this period small amounts of dissociation no doubt took place, as the temperature of the laboratory was between 15° and 20° C.

The results obtained are given in the following table and are set out graphically in Fig. 4.

Solubility of Nickel Carbonyl at Different Temperatures.

Temp.	V. T. of $Ni(CO)_4$ at temp.	Part. P. of gas used	Mgrs. Ni per 100 grs. serum	Calculated for V. T. at temp.
1·1	157	112	16·1	22·57
10·0	219·5	139	10·07	15·71
23·5	364	186	4·66	9·0
35·0	568	209·5	2·71	6·81

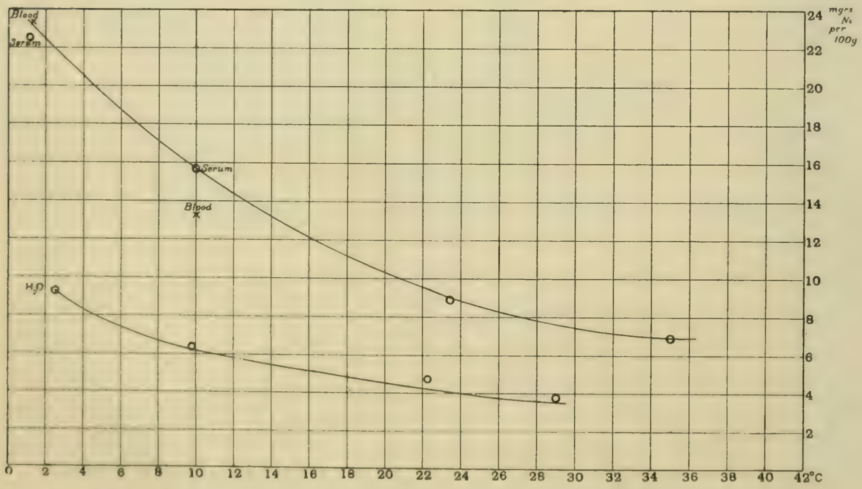

Fig. 4.

The solubility of nickel carbonyl in serum is higher than in water, and at body temperature the solubility in the former is about double that in the latter. In Fig. 4, the solubility curve in water is also plotted upon the same scale for comparison.

Solubility of Nickel Carbonyl in Blood.

At first it seemed not improbable that nickel carbonyl combined with the haemoglobin of the blood in virtue of its carbon monoxide groups, in which case the amount taken up by the blood would be greatly in excess of that dissolved in serum.

Langlois (1891) found that when blood was shaken up with nickel carbonyl the oxygen attached to the haemoglobin diminished by from 50 % to 90 %. He also injected rabbits with from 0·25 to 0·3 gr. of nickel carbonyl subcutaneously. The animals died within from 30 to 35 minutes. No oxygen was found attached to the haemoglobin after death. Lastly, he injected 0·3 gr. of nickel carbonyl into dogs, and obtained a great reduction of the oxyhaemoglobin. He therefore came to the conclusion that the oxygen was replaced by nickel carbonyl.

In an unpublished experiment, Shields was able to recover nickel carbonyl from blood by means of the gas pump. He concluded from this experiment that nickel carbonyl formed a chemical combination with a constituent of the blood.

In examining the behaviour of nickel carbonyl in blood it was thought advisable to proceed by carrying out parallel experiments with serum and blood. For this purpose, a litre bolt-head flask was fitted with a rubber cork, having four holes, to connect (1) with the filter pump, (2) with the mercury gauge, (3) with the nickel carbonyl burette and (4) with a T-shaped tube, leading to two equal 300 c.cm. flasks, one of which contained 100 c.cm. of blood and the other 100 c.cm. of serum. Each of the latter was provided with a capillary glass tube for removing samples of fluid. The flasks were exhausted, the serum and blood boiled vigorously *in vacuo* and the whole apparatus washed out four times with hydrogen, and again exhausted. Nickel carbonyl was run into the bolt-head flask until the mercury registered a pressure nearly equal to the vapour tension of nickel carbonyl at the temperature employed.

After a sufficient time had elapsed to ensure complete solution, samples of each were withdrawn into exhausted bottles and dealt with as in the previous experiments. While one sample was being analysed, the other was kept at 3° C. in the cold room. As the displacing stage occupied some 2 to 2½ days, some dissociation possibly took place, even at the low temperature at which the flasks were kept. After the fluids were freed from nickel carbonyl, they were evaporated to dryness, ashed and the nickel content determined.

The results of two experiments are as follows:

1. Conducted at a temperature of 1·1° C.

Pressure of hydrogen before admitting $Ni(CO)_4$ ... 15·5 mm. Hg.
Pressures when equilibrium was attained ... ... 127·5 mm.

Samples of the liquids were drawn off after 45 minutes. The nickel carbonyl in solution was determined as before.

The blood was dealt with first and yielded 17·35 mgrs. of nickel per 100 grs. which would correspond to 23·42 mgrs. of nickel per 100 grs. at the full vapour pressure of nickel carbonyl for the temperature employed.

The serum yielded 16·1 mgrs. nickel per 100 grs. or 22·57 mgrs. of nickel per 100 grs. calculated for the vapour pressure of nickel carbonyl at 1·1° C.

The blood further contained 2·64 mgrs. nickel in some form not displaceable with hydrogen and the serum contained 8·71 mgrs.

The following table expresses the result obtained, as mgrs. nickel per 100 grs.:

	Ni as $Ni(CO)_4$ mgrs.	Ni in solution mgrs.	Total mgrs. Ni
Serum	16·1	8·71	24·81
Blood	17·35	2·64	19·99

2. Conducted at a temperature of 10° C.

Pressure of hydrogen before admitting $Ni(CO)_4$ ... 25 mm. Hg.
Pressure when equilibrium was attained ... ... 164 mm.

Samples were taken after 45 minutes. The serum was first dealt with, and the results were as follows:

	Ni as $Ni(CO)_4$ mgrs.	Ni in solution mgrs.	Total mgrs. Ni
Serum	10·07	8·27	18·24
Blood	8·53	11·32	19·85

Since the fluid dealt with first in each case gave higher results, it is probable that the amount of nickel carbonyl found in the second fluid was too low on account of dissociation having taken place. One is therefore justified in concluding that at 1·1° C. and 10° C., the amount of nickel carbonyl taken up by blood is about the same as that by serum, and that the values, calculated for the full vapour pressure of nickel carbonyl at these temperatures, are about 23 and 15 mgrs. nickel per 100 grs. respectively. It is not obvious why the two results should be so nearly the same, for the physical and chemical properties of blood and serum differ very considerably.

It is therefore unnecessary to come to the conclusion, as Langlois and Shields have done, that a chemical combination between haemoglobin and nickel carbonyl is formed, as the total quantity of nickel carbonyl which blood is capable of taking up can exist in solution in serum. The oxygen of the haemoglobin is of course replaced by any carbon monoxide which may be liberated by dissociation of the nickel carbonyl. Langlois used quantities of nickel carbonyl which would yield sufficient carbon monoxide after dissociation to saturate the haemoglobin.

On the Properties of the Substance formed by the Dissociation of Nickel Carbonyl in Water in the Presence of Air.

When nickel carbonyl is dissolved in water and dissociation takes place, the condition of the nickel depends on the presence or absence of oxygen and carbon dioxide. In the entire absence of both of these, a precipitate of metallic nickel is formed, which can easily be removed by sedimentation or by filtration. When air is present, the nickel appears as a fine greenish white precipitate in a turbid fluid.

The precipitate is probably a hydrated sub-carbonate (Mond), but the exact composition has not been determined. The turbidity of solutions in which decomposition has occurred cannot be removed by passing the liquid through a paper filter, but when it is passed through a Berkefeld filter a clear filtrate is obtained, which, however, contains nickel in solution. The maximum quantity of nickel which was found in water after dissociation of nickel carbonyl had taken place and the carbonyl and undissolved salts had been got rid of by filtration through a Berkefeld filter was 3·47 mgrs. nickel per 100 c.cm. of water.

The properties of this nickel solution have been compared with those of nickel hydrate and carbonate. The differences are best understood by placing the behaviour of each side by side, and, for the sake of brevity, the product of dissociated nickel carbonyl will be referred to as *dissociation product.*

	Solubility	Filtration	Effect of heat	Effect of addition of Electrolytes (NaCl)
Ni $(OH)_2$	Practically insoluble (less than $\frac{1}{1000}$ mgr. in 10 c.cm. of water).	When water is shaken up with Ni $(OH)_2$ and passed through any filter paper, the filtrate is quite clear.	Unaltered as regards solubility after boiling.	No solution exists.
$NiCO_3$	Is soluble to the extent of 0·48 mgr. Nickel per 100 c.cm. of water at room temperature (18 to 23° C.).	When water is shaken up with Nickel Carbonate, the filtrate is only clear if a close filter is used.	Boiling deposits a trace of precipitate containing Nickel. The filtrate contains the greater bulk.	Electrolytes do not precipitate the salt from solution.
Dissociation Product	Is dissolved to the extent represented by 3·47 mgrs. Nickel per 100 c.cm. water at room temperature.	When water is shaken up with Nickel Carbonyl and the Carbonyl is removed, the liquid can only be cleared of its turbidity by passing through a Berkefeld filter.	From the solution of *Dissociation Product*, the Nickel is almost completely precipitated by boiling. The precipitate can be completely removed by filtering through paper, leaving the merest trace of Nickel in solution.	The addition of Electrolytes to solution of *Dissociation Product* precipitates some of the Nickel. When a saturated solution of NaCl is added, about 25 °/₀ of the Ni is precipitated, while when $(NH_2)_2 SO_4$ is added, rather less is thrown out of solution.

Nickel hydrate is slightly soluble in its mother liquor, *i.e.* sodium hydrate; nickel carbonate is more soluble in its mother liquor, *i.e.* sodium carbonate, and the fluid becomes slightly turbid, which turbidity can only be removed by filtration through a close filter paper.

When carbonic acid is passed through a solution of nickel carbonate, and the liquid filtered through a close filter paper, the solubility is found to be slightly diminished (0·41 mgr. against 0·48 mgr. nickel per 100 c.cm. of water).

After standing for a few days, the solution of *dissociation product* becomes opalescent, and after the 5th day or later a fine precipitate separates out. This precipitate contains nickel.

The solution of *dissociation product* was subjected to dialysis and it was found to pass through the membrane in 36 hours. 74 c.cm. of a solution containing 1·1 mgr. nickel per 100 c.cm. were placed inside the skin and 1500 c.cm. of water were placed outside. The dialysate contained 0·96 mgr. of nickel. A solution of *dissociation product* containing 0·15 mgr. in 50 c.cm. was dialysed for 12 hours with 800 c.cm. of water. At the same time, a solution of nickel carbonate of the same strength was also subjected to the same procedure. The latter was found to have dialysed completely, while the dialysis in the former was not complete. The dialysate contained 0·03 mgr. of nickel, but the fluid inside the membrane only contained 0·01 mgr., so that the remainder was absorbed by the membrane. A solution of *dissociation product* containing 0·41 mgr. per 100 c.cm. was next dialysed for seven hours. It was found that roughly one-third of the same strength solution of nickel carbonate passed through the membrane in this time, the rest being found in the fluid inside the skin. In the case of the *dissociation product* although about 0·02 mgr. of nickel was still in the fluid in the skin, only a mere trace could be found in the dialysate. The greater part of the nickel had therefore been absorbed into the membrane.

Solubility of Dissociation Product *in Serum and its Relation to the Proteid Content.*

Serum was saturated at 35° C. with nickel carbonyl (*a*) in the absence of air and (*b*) in the presence of air, and was then subjected to various investigations. The serum in the former case was boiled *in vacuo*, but this is insufficient to remove all the carbon dioxide. The nickel carbonyl having been removed, the serum was filtered through a Berkefeld filter. The resulting filtrate was quite clear, but the colour varied in consequence of concentration behind the filter.

1. Experiments conducted with serum which had been boiled *in vacuo* and the oxygen removed.

Various samples were analysed for nickel and also for nitrogen (Kjeldahl).

The results were as follows:

	Mgrs. Ni per 100 grs. serum	N per 5 c.cm., expressed as $\frac{n}{10}$ NH_3
1.	2·98	13·7
2.	3·1	18·7
3.	2·18	14·7
4.	1·3	4·7
5.	2·48 (?)	5·2

The figures show no proportion between nickel and nitrogen, so that a chemical compound is excluded.

2. Experiments conducted in the presence of air.

Serum, serum diluted with water, and water were saturated with nickel carbonyl, kept at 35° C. for one hour and then placed in the cold room for 20 hours. The nickel carbonyl was driven off by bubbling air through the liquids, and the liquids were passed through a Berkefeld filter. Samples ashed gave the following values.

1.	Pure Serum	contained	13·33	mgrs.	Ni	per 100 grs.
2.	Serum + water (1 : 1)	,,	7·75	,,	,,	,,
3.	,, ,, (1 : 2)	,,	7·25	,,	,,	,,
4.	,, ,, (1 : 4)	,,	6·19	,,	,,	,,
5.	Pure water	,,	0·72	,,	,,	,,

Here again no ratio between nickel and nitrogen (dilution of serum) is to be found.

Dialysis.

Serum treated as before in the absence of oxygen was subjected to dialysis for 24 hours.

1. 15 grams of Serum inside the skin and 1400 c.cm. H_2O outside.

Dialysate	contained	0·3	mgrs. Nickel.
Fluid inside the skin	,,	1·4	,,

2. 25 c.cm. of Serum and 1315 c.cm. Water.

Dialysate	contained	1·57	mgrs. Nickel.
Fluid inside the skin	,,	0·63	,,

3. 40 c.cm. of Serum and 1500 c.cm. of Water.

Dialysate	contained	2·65	mgrs. Nickel.
Fluid inside the skin	,,	1·22	,,

The serum in 2 and 3 was not quite fresh and allowed some coagulable material to pass through the membrane, but the results show that the nickel is only partially dialysable.

Discussion of the Condition in which Nickel exists in Serum after Dissociation of Nickel Carbonyl has taken place.

The nickel derived from the dissociation of nickel carbonyl in serum in the presence of oxygen and carbon dioxide exists partly in solution and partly as a finely divided precipitate. The latter can only be removed by filtering through a Berkefeld filter. The dissolved nickel does not stand in any proportion to the nitrogen of the serum. The maximum quantity found dissolved in serum expressed as nickel was 13·33 mgrs. per 100 grs. of serum. Solutions of this *dissociation product* are only partly dialysable.

The increased solubility in serum as against water must depend either on the presence of inorganic and organic salts, or on the presence of proteid and other colloidal matter (*e.g.* pigment) or both.

In order to decide to what conditions this increased solubility is due, experiments with nickel hydrate, nickel carbonate and *dissociation product* in various solvents were carried out.

Serum was filtered through a Martin Gelatin filter, and a filtrate was obtained which practically represented the salts of serum dissolved in the water of serum. 100 c.cm. of this solution dissolved 5·04 mgrs. of nickel as *dissociation product.* The same quantity of the solution dissolved 4·15 mgrs. of nickel in the form of nickel carbonate.

The solutions in the salts of serum solution however became turbid after two days. The turbidity could not be removed by filtration through the closest filter paper.

A fresh solution of sodium phosphate ($Na_2HPO_4 + 12H_2O$) containing 0·25 gr. to the litre was next used, as representing the minimum value of phosphates in serum (Hammersten gives this at 0·05 gram pentoxide of phosphorus (P_2O_5) per litre). The solution was saturated with carbon dioxide to correspond more nearly to the condition of serum. It dissolved 2·45 mgrs. of nickel as *dissociation product* and 12·3 mgrs. of nickel as nickel hydrate per 100 c.cm.

Nickel carbonate was also shaken up in the phosphate solution and was found to be practically insoluble.

The solubility of *dissociation product* in solutions of sodium carbonate is approximately the same as that in water.

Since serum dissolves *dissociation product* to the extent of 13·3 mgrs. of nickel per 100 grs. of serum, and whereas water solutions of phosphate of sodium and solutions of the salts of serum dissolve considerably less, there is a balance of dissolved nickel yet to be accounted for.

Serum dissolves freshly precipitated nickel hydrate.

Nickel hydrate freshly precipitated was added to serum, well shaken up at room temperature and filtered through a close filter paper. The filtrate which was quite clear contained 22·5 mgrs. of nickel per 100 c.cm. The serum was not freed from oxygen or carbon dioxide. The unashed filtrate gave the dimethylglyoxime reaction. Since serum to which nickel sulphate and an excess of potassium hydrate are added (*i.e.* potassium nickel albuminate) does not give this reaction, one can deduce that the nickel therefore existed in the filtrate as free ions.

The solubilities of nickel hydrate, nickel carbonate and *dissociation product* are given in the following table for comparison. The solubility of *dissociation product* is inconstant, and depends upon the temperature at which the nickel carbonyl is dissociated. The lower the temperature at which dissociation takes place, the greater is the amount of material which goes into solution in the water.

The figures given are therefore only to be regarded as maximum values obtained.

	H_2O	Na_2HPO_4	Salts of serum sol.	Serum
$Ni(OH)_2$	0	12·3	—	22·5
$NiCO_3$	0·48	0	4·5	16·03
Dissociation Product	3·47	2·45	5·04	13·3

It was thought that if the proteid material combined with nickel, this compound might be thrown down by precipitating the proteids with ammonium sulphate.

25 c.cm. of serum in which *dissociation product* was dissolved was used. Ammonium sulphate was added to this to produce a 33 % saturation. The precipitate was collected and ashed and the ashed material is called (*a*) below. Next, sufficient ammonium sulphate was added to the filtrate to produce a 50 % saturation. The ashed precipitate is called (*b*). The filtrate was saturated with solid ammonium sulphate. The precipitate ashed is called (*c*). The filtrate of this was boiled and the precipitate and filtrate ashed are called (*d*) and (*e*) respectively.

(*a*)	Containing	0·12	mgrs. Ni	or 0·49	mgrs.	Ni	per 100 grs.
(*b*)	,,	about 0·01	,,	or 0·04	,,	,,	,,
(*c*)	,,	0		or 0			
(*d*)	,,	0		or 0			
(*e*)	,,	1·27	,,	or 5·1	,,	,,	,,

This experiment was further compared with the following:

Serum was shaken up with nickel carbonate and filtered through a close filter paper. The filtrate was quite clear. Ammonium sulphate was then added as before.

The results were as follows:

(*a*)	Contained	6·7	mgrs.	Nickel	per 100 grams	Serum.
(*b*)	,,	4·7	,,	,,	,,	,,
(*c*)	,,	0·9	,,	,,	,,	,,
(*d*)	,,	0	,,	,,	,,	,,
(*e*)	,,	4·9	,,	,,	,,	,,

In the case of *dissociation product* the small fraction thrown down may have been due to the action of electrolytes, but the reason why nine-tenths should remain in solution is not clear. In the second case, while the action of electrolytes cannot be excluded, the results seem rather to suggest that a part of the nickel had entered in combination with the proteid.

The facts that when in aqueous solution, nickel is precipitated by boiling, and by adding electrolytes, and that at a certain stage of dialysis, adsorption into the membrane takes place, and also that the difficulty of obtaining a constant strength of the aqueous solutions may be due to the substance not being in ordinary solution, are strongly in favour of the view that *dissociation product* passes into colloidal solution.

The increased solubility of *dissociation product* in serum can also be explained on this view. The influence of serum in keeping metallic precipitates in colloidal solution is well known. When copper sulphide, for example, is formed in serum, it does not precipitate in the usual way but forms a colloidal solution.

The increased solubility of *dissociation product* in serum can be explained in this way. At present, however, it is impossible to exclude the possibility of a proteid combination, but this would at most only affect a small proportion of the dissolved nickel. That the major part of the nickel is not combined with the proteid has already been shown.

To sum up, nickel carbonyl dissociates in the presence of oxygen and carbon dioxide into carbon monoxide and a nickel compound, which is probably a hydrated basic carbonate. This substance is slightly soluble in water (0·0035 °/₀) and considerably more soluble in serum (0·0133 °/₀). When this substance is formed in water or in serum it exists partly in a state of extremely fine division, and this causes a turbidity of the liquid to appear. The particles are so small that filtration through a Berkefeld filter is necessary to clear the liquid.

A comparison with nickel hydrate and nickel carbonate shows that the substance obtained from the dissociation of nickel carbonyl is not identical with either of these, as they exist when precipitated in the ordinary way. The solutions in water and in serum appear to be colloidal solutions. From the aqueous solutions the substance can be precipitated by heat, and to a certain extent by the action of electrolytes, and a precipitate forms spontaneously after standing for some days.

The solubility of this substance in solutions of the salts of serum and in solutions of sodium phosphate does not account for its relative' high solubility in serum. There is no evidence of the existence proteid combination.

SUMMARY AND CONCLUSIONS.

1. Nickel carbonyl is a highly toxic compound.

2. Dissociation of nickel carbonyl takes place rapidly at the temperature of the body in the presence of air, moisture and carbon dioxide.

Nickel carbonyl also dissociates at the temperature of the body in the absence of air, moisture and carbon dioxide, but not to the same extent as in the presence of these owing to the fact that a reversible action occurs between the nickel and the dissociated carbon monoxide.

3. Nickel carbonyl is soluble in water to the amount of 2·36 c.cm. of vapour (*i.e.* 6·43 mgrs. of nickel) per 100 grs. at 9·8° C.

The amount dissolved in water is directly proportional to the pressure, when the temperature is constant.

The solubility in water diminishes as the temperature is raised.

4. The solubility of nickel carbonyl in serum and in blood is about $2\frac{1}{2}$ times greater than that in water. At 10° C. 100 grs. of serum dissolves 5·98 c.cm. of nickel carbonyl vapour (*i.e.* 15·71 mgrs. of nickel).

5. When nickel carbonyl is brought into contact with water, serum, blood and other liquids, if oxygen and carbon dioxide are present, dissociation occurs and a substance is formed, probably a hydrated basic carbonate of nickel, which is slightly soluble, but also forms a very fine precipitate, which renders the liquid turbid. The liquid can only be cleared of the turbidity by passing it through a Berkefeld filter.

6. Water dissolves about 0·0035 °/₀ of the product of dissociated nickel carbonyl (reckoned as nickel) at 18° C. About the same amount is dissolved in 10 °/₀ solutions of sodium carbonate.

The solubility of this product of dissociated nickel carbonyl is greater in serum than in water. Serum dissolves about 0·0183 °/₀ (reckoned as nickel). A solution of the salts of serum, under the ame conditions, dissolves 0·005 °/₀ (reckoned as nickel). Solutions of sodium phosphate of approximately the same strength as the phosphates in serum, dissolve 0·0025 °/₀ (reckoned as nickel).

7. Nickel hydrate is insoluble in water but is soluble in serum to the extent of 0·0225 gr. nickel per 100 grs. serum, and in sodium phosphate solutions to the extent of 0·012 °/₀ (reckoned as nickel).

8. Nickel carbonate is soluble in water to the extent of 0·0005 °/₀ (reckoned as nickel), and in serum to an amount, corresponding to 0·016 gr. °/₀ of nickel. Salts of serum dissolve 0·0045 gr. °/₀ of

nickel carbonate reckoned as nickel. Nickel carbonate is insoluble in sodium phosphate solutions.

9. *Dissociation product* in aqueous solution is precipitated by boiling, and to a certain extent by the addition of electrolytes. Some of it is thrown out of solution spontaneously on standing for some days, whereas solutions of nickel carbonate are not precipitated by either of the above means.

10. Solutions of *dissociation product* dialyse more slowly than solutions of similar strength of nickel carbonate, and at a certain stage in the dialysis the greater part of the nickel is adsorbed into the membrane.

11. No constant ratio exists between the nickel and the nitrogen contents of solutions of *dissociation product* in serum of different strengths.

12. When the proteids of the serum, containing the product of dissociated nickel carbonyl in solution, are precipitated by ammonium sulphate, nine-tenths of the nickel remains in solution. One-tenth is precipitated by adding sufficient ammonium sulphate to produce one-third of saturation, and about an additional one-hundredth by producing one-half of saturation.

13. The product of dissociated nickel carbonyl when dissolved in serum is only incompletely removable from solution by dialysis.

The product of dissociated nickel carbonyl is therefore not identical with nickel carbonate or nickel hydrate, and appears to exist in a condition of colloidal solution.

14. The poisonous properties of nickel carbonyl do not depend, as was at first supposed, on the carbon monoxide of the compound.

15. Nickel carbonyl when mixed with air and inhaled by an animal, cannot be absorbed as such, as it becomes split up into the nickel containing substance (? hydrated basic carbonate of nickel) and carbon monoxide, before or soon after reaching the alveoli of the lungs.

16. The poisonous effects of nickel carbonyl are entirely due to the nickel of the compound. The peculiar toxicity of the compound is due to the fact that being introduced in a gaseous form and that t nickel is deposited as a slightly soluble compound in a very fine of subdivision over the immense area of the respiratory surface

In Part II. the passage of the nickel through the deposition on the respiratory surface to its ultimate followed out.

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ON VARIATIONS OF THE MENINGOCOCCUS AND ITS DIFFERENTIATION FROM OTHER COCCI OCCURRING IN THE CEREBRO-SPINAL FLUID.

By J. A. ARKWRIGHT, M.D.

(*From the Lister Institute of Preventive Medicine.*)

Weichselbaum (1887) first described his *Diplococcus intracellularis meningitidis* (meningococcus) as having been cultivated within a few hours after death from the brain or meninges of six cases of acute cerebro-spinal meningitis. These cases occurred during the years 1885—1887 at Vienna, when no regular epidemic existed. Cases had however occurred from time to time, and there was an epidemic in part of Lower Austria in 1886. The youngest case was 14 years old. He described the micrococcus as being of a wide gonococcus-like shape, occurring in pairs or fours, not in chains, negative to Gram's stain. It failed to grow at 20° C., but grew well on agar at 37° C. almost exclusively on the surface. The growth was viscid, the colonies were often confluent and appeared finely granular when slightly magnified. The organisms required sub-culture every few days to ensure success, were easily killed by drying, and showed very little growth in broth.

Jaeger (1895) described a diplococcus associated with an epidemic of cerebro-spinal meningitis. He stated that it was similar to Weichselbaum's organism but that after a short period of cultivation in artificial media it changed its character, becoming Gram-positive, and occasionally grew in short chains and became resistant to drying.

Councilman, Mallory and Wright (1898) found Weichselbaum's diplococcus in an epidemic in Massachusetts, and confirmed the persistence of its Gram-negative characteristics and its susceptibility to drying.

Still (1898) cultivated the meningococcus from a series of cases of posterior basic meningitis, and found that it agreed very closely with Weichselbaum's description, but that it was a little longer-lived in cultures, and grew rather more readily on artificial media.

Albrecht and Ghon (1903) appear to have proved that Jaeger's meningococcus is another organism which appeared in his cultures, although he probably found the true meningococcus of Weichselbaum in his original preparations.

Various statements have been made about the resistance of the meningococcus to drying. Germano (1897) stated that meningococci mixed with dust and dried lived 80—90 days, but he was working with Jaeger's strain, which is not now regarded as the true meningococcus. Councilman, Mallory and Wright (1898) dried meningococci on sterile paper in Petri dishes and placed these in a dark drawer at room temperature. They succeeded in obtaining cultures from the dried material after 60 hours but failed to do so after 72 hours. Albrecht and Ghon (1903) dried a meningococcus emulsion on coverslips and kept them for 24 hours at room temperature in the dark. After this the cocci were dead. Bettencourt and França (1902) found meningococci alive after six hours, but not after nine hours' drying on cotton threads at room temperature in the dark.

v. Lingelsheim (1906), during the great epidemic in Upper Silesia, found that material obtained by lumbar puncture or post-mortem or from the nose, yielded meningococci more frequently if the specimen was received and cultures were made at the laboratory within a few hours of its removal from the body or the death of the patient.

In the case of 31 post-mortem specimens where special precautions were taken to promote rapidity of transit, the meningococcus was isolated from every one. This observer carried out experiments showing that the meningococcus is not affected by diffuse light though killed by direct sunlight. He is also of the opinion that cultures live a shorter time at low temperatures only because they do not grow and the individual life of the coccus is always short. He found that the meningococcus would survive a temperature of $-10°$ C. or $-20°$ C. for two hours.

It has been stated that the meningococcus is especially susceptible to cold, but the above experiments disprove this.

v. Lingelsheim expresses the opinion that the Gram-negative cocci found by him inside leucocytes in cerebro-spinal fluid in cases of meningitis were always true meningococci. He tested the reactions of cultures of various Gram-negative cocci grown on sugar-containing media, and found that the meningococcus produced acid on dextrose and maltose, but not on laevulose, galactose, mannite, dulcite, cane sugar, lactose or inulin. Indeed he makes the production of acid from laevulose a proof that the organism is not a true meningococcus, but as he used solid media and took the reaction after 24 hours as his criterion his results are hardly comparable with that of Gordon. The different reactions of cultures and Gram-negative cocci in broth containing various carbohydrates as given by Dunn and Gordon (1905) are detailed in a paper by me (on the occurrence of *Micrococcus catarrhalis* and its differentiation from other Gram-negative cocci) in this *Journal* (Jan. 1907).

C. Fraenkel (1905) found that meningococci made a feeble growth on gelatin at 22° C., but not at 20° C.

Other cocci found in the cerebro-spinal fluid drawn off by lumbar puncture, are of special interest on account of the difficulty there may be in differentiating them from the meningococcus, and also because some of them occur associated with the meningococcus when this organism may nevertheless be considered the true or chief cause of the disease. The pneumococcus has long been known to be a cause of primary meningitis. Cases of this nature are described by Weichselbaum (1887). The pneumococcus is not infrequently associated with the meningococcus.

v. Lingelsheim (1906) enumerates the following organisms in addition to the *Pneumococcus,* as being found by him in the central nervous system in epidemic meningitis, *viz.*:—varieties of *Streptococcus, Staphylococcus, Diplococcus crassus, D. mucosus, D. pharyngis, flavus II,* and *Micrococcus cinereus*. The last three organisms are Gram-negative, but are distinguishable by the changes they produce in sugar-containing media, by their other cultural characters, and by agglutination tests.

Diplococcus crassus is of special interest, as v. Lingelsheim considers it identical with Jaeger's meningococcus. It is partly Gram-positive and partly Gram-negative; even cocci from adjacent colonies varying in the degree with which they retain the stain. It readily produces acid from several carbohydrates, including saccharose and lactose, and an antiserum obtained by injecting it into a rabbit does not agglutinate the meningococcus. v. Lingelsheim attributes certain symptoms in some cases of meningitis to an infection with *Diplococcus crassus,* added to that with the meningococcus.

Intracellular cocci in cerebro-spinal fluid may be the *Staphylococcus aureus,* as in a case recorded by Wright and Archibald (1906).

Agglutination experiments with the meningococcus have been recorded by many writers.

Bettencourt and França (1902) examined by the macroscopic method the agglutinating power of the blood serum of persons who were ill and cocci which morphologically resembled meningococci. Culturally (1) meningococci found but not isolated, (2) Gram-positive cocci, and (3) a Gram-negative short bacillus.

Specimen 7. (*M.* 27.) Cerebro-spinal fluid, from a case of meningitis, which was sent by post and was almost 48 hours on the journey. Broken up cells and no cocci were seen microscopically. Only Gram-positive streptococci and staphylococci were isolated, probably contaminations.

Specimen 8. (*M.* 34.) Cerebro-spinal fluid which was about 20 hours in the post, from a typical case of cerebro-spinal meningitis on 4th day of disease. The fluid was watery in appearance and gave a very small deposit on centrifugalising. Microscopically many polymorphonuclears were seen but no cocci.

In the cultures only Gram-positive staphylococci appeared.

Specimen 9. (*M.* 33.) Lumbar puncture fluid from a typical case of cerebro-spinal meningitis at the 6th day of the disease. The specimen was about 20 hours in the post; no leucocytes nor cocci were seen after centrifugalising. Cultures yielded a Gram-positive staphylococcus and a bacillus resembling the *Bacillus coryzae segmentosus.*

Specimen 10. (Yielding strain *M.* 38.) Lumbar puncture fluid from a child aged 5 suffering from meningitis, which was about 16 hours in post, had a deposit of thick pus which was composed chiefly of polymorphonuclear leucocytes some of which contained a few (1 to 3) large Gram-negative cocci of typical appearance. Meningococci grew freely in cultures and one or two colonies of a *Staphylococcus albus* appeared.

The strains of meningococcus XII, XVII, XVIII, XIX, XXIX, and *M.* 38, when grown on blood agar, formed small faintly milky and raised colonies, which after two or three generations grew on ordinary agar at 37° C. as translucent, very slightly whitish colonies, which were confluent when much crowded, and were slightly brownish or amber as seen by transmitted light. Under a 1-inch objective the colonies were seen to be very finely granular or quite smooth. The growth was rather sticky and slimy. The earlier generations died if not sub-cultured every three days, but later generations if protected from drying by tight plugging of the tube, sometimes lived for one month at 37° C. At room temperature sub-culture was never successful after three days. In ordinary broth at 37° C. growth took place (though very slowly when first isolated), a general turbidity being caused, and later a whitish muddy deposit.

When 1 °/₀ of various carbohydrates was added to peptone water and broth (75 °/₀ of the former, 25 °/₀ of the latter) acid was formed in three days from maltose and glucose, and later from galactose and laevulose in the case of XII, XVII, XVIII and XIX; but XXIX, only produced acid from glucose after prolonged artificial culture and not constantly from laevulose. XXIX never formed acid from galactose. None of these strains produced acid from cane sugar or lactose. The medium became turbid and a whitish ring, adherent to the glass, formed at the upper level of the liquid in seven days.

In no instance was the result altered by adding ascitic fluid or serum to the sugar-broth, though the change was sometimes hastened.

These strains did not grow on gelatin at 22° C., but on one occasion when the incubator was raised to 24° C. for a few hours, a very slow growth of XVIII began and continued at 20° C., liquefying the gelatin at the surface. The purity of this culture was repeatedly proved by sub-culture and microscopic examination. Accordingly I made cultures on gelatin of four (XII, XXIX, XVIII, and XIX) of the strains of meningococcus, strain 4*M.* and several strains of *M. catarrhalis* obtained from the nose, and incubated them at 37° C.; after 10 days the gelatin containing strains XVIII, XXIX and XII remained liquid, but was of a thick syrupy consistency, on cooling to 15° C., whereas those tubes containing XIX, 4*M.* and the different strains of *M. catarrhalis* became quite solid.

It, therefore, appears that some difference exists among strains of meningococcus, in the power of producing acid from sugars and in that of liquefying gelatin.

Those strains isolated from typical posterior basic cases showed the most aberrant characters.

The strain 4*M.*, which was found in pure culture and a single colony isolated from the cerebro-spinal fluid of a case of meningitis, is particularly interesting, in that it agreed in some particulars with the meningococcus, but was certainly not a member of this species, and also because Gram-negative intracellular cocci of very similar shape and appearance to meningococci were seen by Dr W. E. Marshall in polymorphonuclear leucocytes in a smear of the same specimen of cerebro-spinal fluid. Of course these may have been meningococci which did not grow in the culture tubes. Only one colony had grown on the blood agar at the end of 48 hours, and this was indistinguishable from a colony of meningococci in appearance. On sub-culture, however, it grew well on agar, but always discretely, and the colonies were whiter than those of meningococcus. It grew in broth rendering the medium uniformly turbid, and the colonies on agar were not coarsely granular and were moist and rather sticky. An emulsion was easily made and did not agglutinate spontaneously. In these respects 4*M.* resembled the meningococcus very closely, but it grew on gelatin at 22° C. without liquefaction; it failed to produce acid from glucose, maltose or galactose, and an emulsion was not agglutinated by meningococcus serum in dilutions of 1—2 and 1—10. Moreover in a film from the original colony the cocci were rather larger and stained better with methylene blue than it is usual for meningococci to do. It is, therefore, clear that this organism is not the meningococcus. It resembles *Micrococcus cinereus* of v. Lingelsheim in most particulars. (See my paper on *Micrococcus catarrhalis* &c. in this *Journal*, Jan. 1907, Table III shewing cultural differences.)

I made four experiments with regard to the power which the meningococcus has to withstand drying.

Experiment 1. Impressions of blood agar colonies of XXIX and XVIII on coverslips were put into dry sterile tubes and left at room temperature for 2 days, and then transferred to broth and incubated at 37° C. The meningococcus could not be recovered from them.

Experiment 2. Portions of broth cultures of strains XVIII and XIX were dried in sterile Petri dishes (*a*) mixed with sterile sand, (*b*) mixed with sterile violet powder, (*c*) without admixture. The dishes were then placed in an air-pump receiver over strong sulphuric acid and the air exhausted. Samples taken at the end of 24 hours did not contain living meningococci.

Experiment 3. Some of a broth culture of strain XXIX was dried on strips of sterile blotting paper or on sand, over sulphuric acid in the receiver of an air-pump; after three hours the sand looked dry but was still damp; from a sample put into broth and incubated at 37° C. meningococci grew. From a strip of blotting paper taken at the same time and put into broth at 37° C. no meningococci grew. After 24 hours and complete drying of the sand no meningococci could be recovered.

Experiment 4. A broth culture of strain XII poured on dry sterile garden soil in a Petri dish and left at room temperature yielded no meningococci after 20 hours.

To test resistance to cold, agar cultures of four strains of the meningococcus (XII, XVII, XVIII, XIX) were put in the cold room at 1° C. They could be sub-cultured after 72 hours.

In order to further test the susceptibility of this organism to cold, the cerebro-spinal fluid of specimen 10 was placed in the cold room at 4° C., and successful cultures were made after 48 hours, but not after 72 hours. Before this specimen reached me 15 hours had already elapsed since its removal from the body.

This high degree of viability is in marked contrast to that observed in the case of some strains such as those contained in specimens 7 and 8, which performed the same journey but could not be grown when received though similar care and media were used. It seems probable therefore that the rapid death of meningococci in lumbar puncture fluid, which has often been observed, is not due to cold but to some other cause, such as the presence of other bacteria or of harmful substances in the cerebro-spinal fluid as suggested by Flexner (1906).

Agglutination Experiments.

To prepare an agglutinating serum from a rabbit it is necessary to give large and frequent doses of meningococci intravenously for a considerable time, and the serum appears to quickly lose its agglutinating properties if the injections are discontinued.

Agglutinating serum was obtained for me by Dr W. E. Marshall by injecting large quantities of meningococci intravenously into three rabbits; the doses chiefly used were from 2—4 agar tubes, and the injections had to be given for 4—5 weeks before the serum showed much agglutinating power. I used the microscopic method of examination for agglutination.

Rabbit I was injected intravenously with 2-day agar cultures of meningococcus XII, emulsified in a 0·9 % sodium chloride solution and killed at 65° C. for 30 minutes.

TABLE I.

Agglutination with serum from Rabbit I.

		1/2	1/10	1/25	1/50	1/100	1/200	1/500	1/1000	Normal 1/2
March 30	Microscopic									
	XII & XVIII 1½ hrs.	–		–	–					
	Macroscopic									
	XII 24 hrs.			–	–	–				
April 26	Microscopic									
	XII 1 hr.	+	+	sl.		–				–
May 1	Microscopic									
	XII 1 hr.		+++	++	+	++	+	sl.	–	
	XIX ,,		+++	++	+	++	++	+	–	
	XVIII ,,	+++	+++	++	+	+	+	+		
	XVII ,,	+	++	++	+	+	sl.			
	XXIX ,,	+	–	sl.	sl.	–	–			
May 19	XII ,,	+	sl.							
	XXIX ,,			+	sl.					
				1/20						1/2000
May 25	XII ,,	+	+	++		+	++		+	–
	XVIII ,,	+++	+++	+++	+++	+++	+++			
	XXIX ,,	+++	+++	++	++					
	4*M.* ,,	–	–	–						

March 9th. 4 agar tubes injected intravenously.
,, 16th. 7 agar tubes injected intravenously.
,, 30th. Bled : the serum did not agglutinate meningococcus XII nor XVII in a dilution of 1—2.
April 6th. 4 agar tubes injected intravenously.
,, 12th. 3 agar tubes injected intravenously
,, 17th. 3½ agar tubes injected intravenously
,, 21st. 4 agar tubes injected intravenously
,, 24th. Bled : agglutination of meningococci in 1—2 and 1—10 dilutions.
May 1st. Bled : agglutination good 1—200 to 1—500.
,, 9th. 3 agar tubes injected.
,, 18th. 3 agar tubes injected.
Bled.
,, 23rd. Bled.

Rabbit II. Injected intravenously with 2-days' old agar cultures, killed at 65° C. for 30 minutes.

March 9th. 4 agar tubes injected.
,, 16th. 7 agar tubes injected.
April 3rd. Bled : no agglutination.
,, 6th. 4 agar tubes injected.
,, 12th. 3 agar tubes injected.
,, 17th. 3½ agar tubes injected.
,, 21st. 4 agar tubes injected.
,, 24th. Bled : agglutination 1—10.

TABLE II.

Agglutination with serum of Rabbit II.

		1/2	1/10	1/25	1/50
	Microscopic				
April 3	XII 1 hr.	–		–	–
	XVII ,,	–		–	–
April 24	XII	+	+		sl.
	XII with normal serum from rabbit.	–	–		

This rabbit shows the difficulty sometimes encountered in obtaining an agglutinating serum.

Rabbit III. Injected with agar cultures of meningococcus XXIX killed at 60° C.

April 9th. 3½ agar tubes of 48 hours' growth injected.
,, 12th. 3 agar tubes of 48 hours' growth injected.
,, 17th. 4 agar tubes of 3 days' growth injected.
,, 24th. Bled.
,, 30th. 3 tubes of 48 hours' growth injected.
May 8th. 3 tubes of 24 hours' growth injected.
,, 18th. 3 tubes of 24 hours' growth injected.
Bled.

TABLE III.

Agglutination with serum of Rabbit III.

Dilution	1/2	1/10	1/50	1/100	1/200	1/1000	1/2000
9 April 1906 XXIX	–	–	–				
26 April 1906		++	++	++	+		
19 May 1906			++	++	++	+	

One sample of cerebro-spinal fluid which I examined, and which was drawn by lumbar puncture from a child who had been ill about 13 days, agglutinated the corresponding strain (XXIX) in dilution of 1—5, and other strains (XVII and XVIII) in dilutions of 1—100 and 1—50 respectively.

TABLE IV.

Agglutination with cerebro-spinal fluid from the same case from which Strain XXIX was isolated.

Dilution	1/2	1/20	1/50	1/100
XXIX	+	–	–	–
XVII	+	+++	++	+
XVIII	+	++	+	–

The serum of rabbits repeatedly injected with cultures of *M. catarrhalis* did not agglutinate the *meningococcus*, and could not be tested on *M. catarrhalis* on account of the constant spontaneous agglutination of this organism.

The agglutinating power of *M. catarrhalis* serum has been tried by v. Lingelsheim (1906). He found that it never agglutinated the *meningococcus* in higher dilution than 1—50 macroscopically.

Conclusions.

1. Gram-negative cocci obtained from the cerebro-spinal fluid are not always true meningococci, even in cases of meningitis.
2. Slight differences between different races of meningococci occur, especially as regards their growth and activity in sugar media and on gelatin.
3. The meningococcus is not easily killed by cold, therefore its rapid death in lumbar puncture fluid and post-mortem material must be due to some other cause.
4. The means by which the meningococcus is carried from the diseased to the healthy can hardly be such as to involve drying.

I am indebted to the late Dr A. Macfadyen for his help and suggestions, also to Dr A. E. Boycott for his advice and assistance, and to Dr W. E. Marshall for his help in connection with the animal inoculations. I have also to thank Dr Morley Fletcher, Dr Montague Murray, Dr Walter Carr, and Dr M. Macdonald for supplying me with material.

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OBSERVATIONS ON THE BACTERIOLOGY OF AN EPIDEMIC OF DIPHTHERIA IN A SCHOOL,

WITH SPECIAL REFERENCE TO THE VIRULENCE OF THE ORGANISMS WHICH WERE ISOLATED FROM THE CASES.

BY J. A. ARKWRIGHT, M.D.

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IN November 1906 an opportunity occurred to investigate an epidemic of diphtheria occurring at the Duke of York's School, Chelsea. This is a military school of 551 boys, between the ages of 9 and 14 years, and 40 students, or older lads, 17 to 19 years of age. A certain number of both boys and students were affected.

History of the outbreak.

The epidemic of sore throats began about the 26th of August 1906, immediately after the boys had returned from camp at the seaside. Throughout September, October, and the first half of November, cases of sore throat occurred daily with occasional intervals of a day or two. Almost all of the cases presented the clinical features of follicular tonsillitis.

The earliest case which was clinically considered to resemble diphtheria occurred on 20th September, in a boy who had at the time, in addition to sore throat, a free nasal discharge which continued with an interval for at least two months; his nose and throat yielded in November the *Bacillus diphtheriae.*

Usually there was a sudden onset with vomiting and a temperature of 101°—103° F. The illness only lasted four or five days, after which the patient, as a rule, appeared well. In no case was there a rash. No cases of peritonsillar abscess were observed. Altogether 183 boys and students had sore throats.

Albumen was found in the urine of two of the boys who were removed to the Metropolitan Asylums' Board Hospital. But although the urine of many of the patients who remained in the school was examined, in none was albumen found. Paralysis of the fauces was observed in only two cases, and in one of these accommodation was also affected. The general course of the disease was in every case mild, and recovery was uneventful, with the exception given above. All those affected recovered completely.

The epidemic was dealt with in the following manner by Lieut.-Col. Fayrer, the Medical Officer to the Duke of York's School: between 17th and 22nd November 1906, the whole school received a dose of diphtheria antitoxin, 109 boys in whose throats diphtheria bacilli had been found received 1000 units, and the rest 500 units: in addition all those with *B. diphtheriae* were either sent to hospital or isolated.

After the prophylactic injection until the middle of January, only five cases of sore throat occurred, and in none of these was the diphtheria bacillus found. But in one boy in apparent health, a patch of membrane was found on the fauces on 12th December, from which diphtheria bacilli were obtained. The epidemic ended suddenly on the adoption of the plan of isolation of carriers and prophylactic injection.

I first had the opportunity of examining swabs taken from the boys' throats on the 3rd November, and after that I examined all cases of sore throat for *B. diphtheriae.* Swabs taken from the fauces were inoculated the same day on solidified blood serum, and after the tubes had been incubated at 37° C. for about 20 hours, they were examined for *B. diphtheriae.* Its presence or absence was determined by examining films stained by Loeffler's methylene blue.

In order to determine with greater certainty the nature of such strains as were isolated, in addition to observing morphological and cultural characters, experiments with regard to acid production from various carbohydrates, virulence for guinea-pigs, and agglutination were made.

Results of Examinations of Swabs from Throats.

Forty boys were examined whilst actually suffering from sore throat between the 3rd and 18th of November, and of these 15 (= 37 %) yielded *B. diphtheriae*.

On 14th November, Dr Boycott, Dr Marshall and myself, took swabs from the fauces of all the boys in the school who were not in hospital, and on 19th November 33 students were examined in the same way. On these two occasions 537 boys and students were examined and a positive diagnosis of *B. diphtheriae* was made in 118 cases (= 21 °/₀), all of whom, with one exception, appeared to be in good health. In this case a patch of membrane was found on the fauces at the time when the swab was taken. In addition 182 (= 43 °/₀) were found to harbour *B. pseudodiphtheriae* (Hofmann). No attempt was made to count the number of those yielding Hofmann's bacillus in addition to *B. diphtheriae.* The above 182 cases with Hofmann's bacillus were found amongst those not giving *B. diphtheriae,* and the prevalence of Hofmann's bacillus is therefore best indicated by reckoning these cases as a percentage of the 419 cases in which *B. diphtheriae* was not found. Calculated in this way the proportion with Hofmann's bacillus was 43 °/₀.

If we now take the whole school of 591 boys and lads into consideration (including the 40 students of 17—19 years), and the whole time from the beginning of the epidemic in the last days of August 1906 to the middle of January 1907, the following facts emerge:

Total number of boys and students in school = 591,
Total number who had sore throats = 183 or 31 °/₀,
Total number found with *B. diphtheriae* = 136 or 23 °/₀.

Incidence of B. diphtheriae.

Of 40 boys with sore throat when examined ...	15 or 37 °/₀	had *B.*	*diphtheriae.*
Of 183 boys who at some time had sore throats	55 or 30 °/₀	,,	,,
Of 591 boys (*i.e.* whole school)	136 or 23 °/₀	,,	,,
Of 408 boys who did not have sore throats ...	81 or 20 °/₀	,,	,,

Very few examinations were made before the 3rd November.

The boys who suffered from sore throats and those who were proved to harbour *B. diphtheriae* were by no means identical, for although 136 yielded *B. diphtheriae,* only 55 of these had had sore throat.

Characters of the Strains isolated.

Twenty strains of bacilli morphologically resembling *B. diphtheriae* isolated from cases taken haphazard were further examined. Four of these strains were from patients who had clinical symptoms when examined (3 cases of sore throat and one of nasal discharge), the rest were from boys who had no symptoms when examined, but 3 of whom had had sore throats about four weeks previous to the examination.

One of these 20 strains (D 73) morphologically and culturally somewhat resembled *B. coryzae segmentosus*, but a dose of 2 c.c. of a 48-hour broth culture killed a guinea-pig in 4½ days. In addition 4 other diphtheroid strains were isolated; 2 of Hofmann's bacillus (A 12, D 39), and 2 organisms resembling *B. coryzae segmentosus* (G 17, G 36). Certain other strains of diphtheria and diphtheroid bacilli obtained from other sources were subjected to the same tests for the sake of comparison (see footnote to Table I).

Morphology.

As described by other writers, films made from 20-hour cultures on solidified serum and stained by Loeffler's blue showed many variations in the length and structure of the bacillus. All the strains showed beading or segmentation and characteristic grouping.

Three of the strains (B 28, C 49, and Y 11), which were most typical of the long form of the *B. diphtheriae*, were amongst the non-virulent strains. Neisser's stain yielded uncertain results; for instance virulent strains of *B. diphtheriae* (Y 57, St. 28) showed no polar bodies, but diphtheroids (D. 39, Waite) which certainly were not true *B. diphtheriae* had well marked polar bodies.

B. coryzae segmentosus in a film from a 20-hour culture usually appeared as a very short diplococcus-like bacillus, but after 48 hours or more had grown into a long and much segmented form, sometimes with swollen ends. After a few days the beaded forms of the bacillus when stained with Loeffler's blue often closely resembled a chain of streptococci.

None of the 7 strains of non-virulent *B. diphtheriae* isolated in this epidemic resembled those races of diphtheroids described by Gordon (1902) or Graham-Smith (1904) as being related to but not identical with the *B. diphtheriae.*

Acid Production from Sugars.

Liquid media containing 1 % of glucose, maltose, galactose, laevulose, lactose, cane sugar and mannite were used in these tests. A series of tests was made with a medium consisting of 25 % beef broth and 75 % peptone water and litmus. The results are given in Table I. Acid was produced by the strains of *B. diphtheriae* in the presence of all these substances except cane sugar and mannite.

A more complete series was carried out with litmus-peptone-water (2 % Witte's peptone in tap water) as the basis of the medium. This

TABLE I. *Weak peptone broth with 1 °/₀ carbohydrates.*

Bacillus diphtheriae	Glucose	Maltose	Laevulose	Cane sugar	Lactose	Mannite	Galactose	Dextrin	Glycerin	Virulence
St. 28	+	+	+							VV
A 67	+									VV
St. 29	+	+	+		+	–				VV
E 25	+				+					VV
D 3	+				+					VV
G 50	+	+	+		+	–				VV
Y 3	+	+	+		+					VV
A 37	+	+	+			–				V
Y 35	+	+	+		+	–				V
Y 57	+	+	+		+	–				V
G 61	+S	+	+		+					V
E 71	+									V
D 73	+S									V
Bacilli from other sources :										
P. W.										VV
Bl. 2										V
Bl. 4										VV

Bacillus diphtheriae	Glucose	Maltose	Laevulose	Cane sugar	Lactose	Mannite	Galactose	Dextrin	Glycerin	Virulence
E 6	+	+	+	–	+	–				N
St. 33	+	+	+	–	+	–				N
St. 22	+				+					N
B 28	+	+	+	–	+	–				N
Y 11T	+	+	+	–		–				N
C 49	+	+	+	–	+	–				N
St. 17	+S	+	+S	–	+	–				N
B. pseudo-diphtheriae (Hofmann) :										
A 12	–	–	–	–	–	–				N
D 39	–	–	–	–	–	–				N
B. coryzae segmentosus :										
G 17	+S		+S							N
G 36	+S	–	–	–	–	–				N
Other diphtheroids from outside sources :										
Ble.	+									
By.	+									
W.	+									
18 (2)	+S									
21 (1)	+S									

Hiss' medium with 1 °/₀ carbohydrates.

Graham-Smith									
B. diphtheriae	+	+	+	∓?	+	–	+	+	+
B. xerosis	+	–	+				+	–	–
B. coryzae segmentosus	+	–	+	–	–	–	+	–	–
Certain other diphtheroids	+	+	+	–	–	–	+	–	–

Knapp								
B. diphtheriae	+	+		–	+	+	+	+
B. xerosis	+	+		+		+		–

+S = slightly acid after 48 hours. + = acid after 48 hours. VV = fair virulence. V = low virulence. N = non-virulent.

The results recorded by Knapp (1904) and Graham-Smith (1906) are included in this Table.

Sources of B. diphtheriae and diphtheroid bacilli examined.

(1)	P. W.	The toxigenic strain No. 8 of Park and Williams.
(2) (3)	Bl. 2 Bl. 4	Virulent strains of *B. diphtheriae* from Dr Blumenthal of Moscow; the bacilli are of very long form and often branched.
(4)	Ble.	A bacillus from ear discharge in scarlet fever; thick opaque, whitish growth on agar; stained nearly uniformly by methylene blue.
(5)	By.	From discharge from the middle ear. It resembled 'Ble.' macroscopically; when stained by methylene blue it showed regular transverse bands.
(6)	W	From the vaginal discharge of a child. It grew feebly in very small colonies on serum and agar. Uniformly barred when stained by methylene blue.
(7) (8)	18 (2) 21 (1)	Diphtheroids of the type of *B. coryzae segmentosus* from catarrhal noses.

TABLE II. *Peptone water* (2 %) *with* 1 % *carbohydrates.*

Strains of *B. diphtheriae*	Virulence	Glucose	Maltose	Laevulose	Cane sugar	Lactose	Mannite	Galactose
St. 28	VV	+	+	+	–	–	–	+
A 67	VV	+	+	+	–	–	–	+
St. 29	VV	+	+	+	–	–	–	+
E 25	VV	+	+	+	–	–	–	+
D 3	VV	+	+	+	–	–	–	+
G 50	VV							
Y 3	VV	+	+	+	–	–	–	+
A 37	V	+	+	+	–	–	–	+
Y 35	V	+	+	+	–	–	–	+
Y 57	V	+	+	+	–	–	–	+
G 61	V							
E 71	V							
D 73	V							
Strains from other sources:								
P. W.	VV	+	+	+	–	–	–	+
Bl. 2	V	+	+	+	–	–	–	+
Bl. 4	VV							

Strains of *B. diphtheriae*	Virulence	Glucose	Maltose	Laevulose	Cane sugar	Lactose	Mannite	Galactose
E 6	N							
St. 33	N	+	+	+	–	–	–	+
St. 22	N							
B 28	N							
Y 11T	N	+	+	+	–	–	–	+
C 49	N	+	+	+	–	–	–	+
St. 17	N	+	+S	+	–	–	–	+
Strains of *B. pseudo-diphtheriae* (Hofmann):								
A 12	N	–	–	–	–	–	–	–
D 39	N	–	–	–	–	–	–	–
Strains of *B. coryzae segmentosus*:								
G 17	N	+	–	+	–	–	–	+S
G 36	N	+S	–	–	–	–	–	+S
Strains of other diphtheroids from outside sources:								
Ble.	N	+	–	+	+	–	–	+
By.	N	+	–	+	+	–	–	+
W	N	+	–	+	–	–	–	+
18 (2)	N							
21 (1)	N	+	–	–	–	–	–	–

VV = killed guinea-pig in dose of 0·1 c.c.
V = ,, ,, ,, 2·0 c.c.
N = did not kill guinea-pig in dose of 2·0 c.c.

+ = acid.
+S = slightly acid.
– = not acid.

series (Table II) gave uniform results which were different from the previous series (Table I) in that acid was never produced from lactose. In the tubes containing cane sugar and mannite, acid was never produced in either series. The growth was good in all the tubes.

It was thought that the muscle sugar in the beef broth might account for the acidity in the lactose tubes of the first series, although no acid had appeared in the presence of cane sugar or mannite. Accordingly 12 strains were tested again in three different media:

(1) Weak litmus broth as above, without lactose.
(2) Weak litmus broth as above, with 1 °/₀ lactose.
(3) Peptone water with 1 °/₀ lactose.

The result is shown in Table III.

TABLE III.

Bacillus diphtheriae	Virulence	Weak broth, no lactose		Weak broth, 1 °/₀ lactose		Peptone water 1 °/₀ lactose
		2 days	7 days	2 days	7 days	7 days
St. 28	VV	+S	S	+	++	–
A 67	VV	+S	+S	+	++	–
E 25	VV	+	+	+	++	–
D 3	VV	–	–	–	–	–
Y 3	VV	+S	+	+	++	–
Y 35	V	+S	+S	+	++	–
Y 57	V	+S	+S	+	++	–
C 49	N	+S	–	+	+S	–
St. 17	N	+S	–	+	++	–
P. W.	VV	–	–	S	VS	–
Diphtheroids from otorrhoea:						
Ble.	V	–	–	+	++	–
By.	N	–	–	S	–	–

VV = virulent.
V = low virulence.
+S = slightly acid.
+ = acid.
++ = very acid.
– = neutral or alkaline.

The supposition was not found to be justified, for as before, the peptone water with 1 °/₀ lactose remained uniformly neutral; the weak broth without lactose was usually slightly acid after one or two days but later became less acid or alkaline; the weak broth with 1 °/₀ lactose soon became acid and later a strong acid reaction developed in it.

Virulence.

The virulence of each strain was tested by injecting subcutaneously into three guinea-pigs (about 250 grammes) doses of 0·1 c.c., 2·0 c.c., and 2·5 c.c. respectively of a 48-hour culture in alkaline broth, 0·1 c.c. of antitoxin having been previously added to the 2·5 c.c. dose. The animal which received antitoxin along with the culture remained well in every case. The results are given in Tables IV and V below.

TABLE IV. *Virulence for the guinea-pig of the isolated strains.*

	Dose of a 48 hrs. culture in alkaline broth			Occurrence of
Strains	2·0 c.c.	0·1 c.c.	Organism	sore throat
St. 28	†17 hrs.	†36 hrs.	Bac. d.	
A 67	†36 ,,	†36 ,,	,,	P
St. 29	†36 ,,	†36 ,,	,,	
E 25	†12 ,,	†84 ,,	,,	
D 3	†60 ,,	†6 days	,,	
G 50	†84 ,,	†8 ,,	,,	
Y 3	†20 ,,	†10 ,,	,,	E
A 37	†19 ,,	–	,,	
Y 35	†36 ,,	–	,,	E
Y 57	†36 ,,	–	,,	E
G 61	†36 ,,	–	,,	P
E 71	†36 ,,	–	,,	P
D 73	†4½ days	–	,,	
E 6	–	–	Bac. d.	
St. 33	–	–	,,	
St. 22	–	–	,,	
B 28	–	–	,,	
Y 11T	–	–	,,	P
C 49	–	–	,,	
St. 17	–	–	,,	
A 12	–	–	H	
D 39	–	–	H	
G 17	–	–	B. C. S.	
G 36	–	–	B. C. S.	

Bac. d. = *Bacillus diphtheriae.*
H = ,, *pseudo-diphtheriae* (Hofmann).
B. C. S. = ,, *coryzae segmentosus.*
E = at time of examination.

P = previously.
† = death.
– = lived.

E and P in the 5th column refer to the occurrence of sore throat in the patient from whom the strain was isolated.

TABLE V. *Proportion of Virulent and Non-virulent Strains.*

	Killed in dose of				
	0·1 c.c. within	2·0 c.c. within	Glucose reaction	Morphology	Percentage
4 Strains	4 days	48 hours	Acid	Bac. d.	35
3 ,,	10 ,,	4 days	,	,,	
6 ,,	–	4½ ,,	,,	,,	30
7 ,,	–		,,	,,	35
20					

Bac. d. = *Bacillus diphtheriae.*

Of the 4 strains regarded on other grounds as *B. pseudo-diphtheriae* (Hofmann) and *B. coryzae segmentosus*, none caused death.

Of the 20 strains from the school resembling *B. diphtheriae* morphologically, only four killed within 4 days, when a dose of 0·1 c.c. was injected; one in 8 days and one other in 10 days; and six strains killed in a dose of 2·0 c.c. *leaving seven strains* (35 %) *quite devoid of killing power.*

If the 20 strains isolated can be taken as a fair sample of the rest, then 7 % of the boys in the school harboured non-virulent *B. diphtheriae.*

In order to make sure that the defective virulence was not due merely to the sample of broth used or to a weak, but not entirely absent, power of forming toxin, 4 non-virulent strains were grown for 10 days in a special alkaline broth which contained 2 % peptone and which had been found very efficient for making toxin. The same doses as before were injected into guinea-pigs, but in no case did death result.

Agglutination.

To obtain further evidence which should decide the question as to the relationship of the virulent and non-virulent strains of diphtheria bacilli, I applied an agglutination test. For this test I employed the serum of a horse which had been immunised some time before, with the view to testing the value of anti-microbial serum.

In the process of immunisation one race of *B. diphtheriae* was employed, viz. the toxigenic bacillus No. 8 of Park and Williams. At the outset of the immunisation intravenous injections of killed cultures of the bacillus were employed, later the living bacilli were used.

The agglutination results (Table VI) were not uniform for the virulent strains, but the majority of these, 10 in number (including the strains from outside sources), gave positive results. One gave a slight, one a doubtful result, and one was negative; three were not tested.

Of the non-virulent diphtheroids, the two strains of *B. pseudo-diphtheriae* (Hofmann) and the two strains 'Ble.' and 'By.' from ear discharges gave negative results.

Two non-virulent diphtheroids (W and 18 (2)) gave doubtful results; one (21 (1)) gave a negative result.

Of six non-virulent diphtheria bacilli from this epidemic, four gave slight positive, and two negative results.

The agglutination tests indicate differences in the races of virulent diphtheria bacilli, and also a divergence of the non-virulent strains from the majority of virulent strains.

TABLE VI. *Agglutination Macroscopic.*

Strain	Virulence	Immune serum				Normal horse serum				Salt soln.
		1/20	1/50	1/100	1/200	1/20	1/50	1/100	1/200	
St. 28	VV	+ + +	+ +	+ +		+ +	+	S	S	S
A 67	VV	+ + +	+ + +	+ + +	+ +	+ +	S	S	S	S
St. 29	VV	+ + +	+ +	+ +	+ +	+	S	S	–	–
E 25	VV	+ + +	+ +	+	S	+	–	–	–	–
D 3	VV	+	+	S	–	+	–	–	–	+
G 50	VV	+	+ S	+ S	–					–
Y 3	VV	+ +	+ +	+	+	+ +	+	+	S	S
A 37	V	+ + +	+ +	+ +	+	+ +	+ +	S	S	+ + +
Y 35	V	+ + +	+ + +	+ +	+	+	+	+	+	+ S
G 61	V	+ + +	+	S	S	+	S	S	S	–
E 71	V									
D 73	V									
From other sources:										
P. W.	VV	+ + +	+ S	S	S	+ S	S		S	
Bl. 2	V	+ + +	+ + +	+ + +	+ +	S	S	S	–	–
Bl. 4	VV	+ + +	+ + +	+ + +	+ +	S	S	VS	VS	

Strain	Virulence	Immune serum				Normal horse serum				Salt soln.
		1/20	1/50	1/100	1/200	1/20	1/50	1/100	1/200	
E 6	NV	+ + +	+	+	–	+ +	+	–	–	–
St. 33	NV	+ + +	+ + +	+	S	+ + +	S	S	S	S
St. 22	NV	+ +	+	S	–	+	S	–	–	
B 28	NV	+ +	–							
Y 11	NV	+ + +	+ + +	+ +	+ +	+ + +	+ +	S	S	S
C 49	NV	+ +	+ + +	+ + +	+	+ + +	+ +	+ +	+	+
A 12 H	NV	–	–	–	–					–
D 39 H	NV	–	–	–	–					–
G 17										
G 36										
St. 17	NV	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Strains from other sources not *B. diphtheriae.*										
Ble.	NV	–	–	–	–	–	–	–	–	
By.	NV	+ S	+ S	–	–	+	–	–	–	
W	NV	+ +	+ +	+	+	+	+ S	+ S	+ S	+ S
18 (2)	NV	+ +	+	+	S	+	+	+ S	S	–
21 (1)	NV	+ + +	+ + +	+ +	+ +	+ + +	+ + +	+ +	+ +	+ +

+ + +, + +, +, + S, S and VS = different degrees of sedimentation in the tubes.
+ + + = complete sedimentation.
– = no sedimentation.

The Occurrence of Bacillus fusiformis and Spirochaetes.

Another interesting feature of the epidemic was the frequent occurrence in the cases of sore throat of the *B. fusiformis* and of spirochaetes. These organisms were seen in smears made direct from the throat. They were found chiefly in cases in which *B. diphtheriae* was also present.

Of 45 cases of sore throat examined 20 showed *B. fusiformis* and 14 also yielded spirochaetes, both of which were often present in very large numbers in smears made at once from swabs taken from the fauces. Of the 16 cases of clinical diphtheria in which *B. diphtheriae* was found, 11 had also *B. fusiformis* and 10 spirochaetes as well as *B. fusiformis*. If the swabs had been taken a few hours or allowed to dry before the smears were made, *B. fusiformis* and spirochaetes were seldom found, and if found, were present in small numbers.

The frequent occurrence of *B. fusiformis* and spirochaetes on the fauces of patients suffering from diphtheria has been described by Priestley (1906) and Leiner (1906). Leiner considers the coexistence of *B. diphtheriae*, fusiform bacilli and spirochaetes to be quite common, and says that they are found associated mainly in two distinct groups of clinical diphtheria: the one, of very severe—the so-called septic type—the other of milder character with slight general symptoms.

Remarks.

The large number (21 °/₀) of boys in the school who were found to harbour *B. diphtheriae* in the fauces, and the large proportion of the strains of bacillus which proved to be either non-virulent or of low degree of pathogenicity for guinea-pigs, is remarkable. Non-virulent strains were estimated to be present in 7 °/₀ of all the boys. The presence of bacilli in the throats of 4 to 10 °/₀ of apparently healthy children, in connection with outbreaks of diphtheria, has been recorded by Graham-Smith, Thomas, Ustvedt, Pennington, and others.

Graham-Smith (1904) during an epidemic of diphtheria at Cambridge found diphtheria bacilli in the fauces of 3·9 °/₀ of the children who were contacts (in 2·6 °/₀ virulent and in 1·3 °/₀ non-virulent strains) and taking the notified cases and contacts together, in 8·2 °/₀ (in 6·4 °/₀ virulent, in 1·8 °/₀ non-virulent) of the children examined.

Thomas (1905) found from the examination of those children in London schools, who without obvious symptoms of diphtheria were suspected of spreading the disease, that 6·7—7·3 °/₀ harboured diphtheria bacilli.

Ustvedt (1906) found that in infected schools in towns, bacilli morphologically indistinguishable from diphtheria bacilli, were obtained from 4·5 % of the children. In an uninfected country district, none could be found amongst the school children; they were however present in the fauces of 14·21 % of those in close contact with patients.

Pennington (1907) examined the fauces of a large number of apparently healthy school children in Philadelphia. He obtained bacilli resembling *B. diphtheriae* from about 10% of the children and of the strains isolated 35—50% showed no virulence for guinea-pigs, and 14% killed guinea-pigs fairly promptly, whereas 30% he considers showed some pathogenicity, but were of attenuated virulence for guinea-pigs.

Where the observations have been made from cases distributed over a town or district, no relation has been established between the severity of an attack of diphtheria and the virulence of the bacilli for guinea-pigs (Smith and Walker (1896), and Richmond and Salter (1898)).

Cobbett (1901) and Graham-Smith in an epidemic in which cases of very different degrees of severity occurred, found that the strains isolated were of a singularly uniform degree of virulence. They obtained no evidence in favour of the possibility of attenuation of the bacillus.

There is more likelihood, however, that an epidemic within a small area, as in the present case, may have originated from a single strain of bacillus. The low degree of virulence of most of the strains which were pathogenic for guinea-pigs and the high percentage of non-virulent strains associated with a large number of cases of a very mild character can be best interpreted by the assumption that the outbreak in this school was due to an attenuated strain of the *B. diphtheriae.*

The agglutination test which I have used has yielded variable results with virulent and non-virulent strains, but on the whole has tended to accentuate the difference between the virulent and the non-virulent. Other observers have used agglutinating sera for examining strains of *B. diphtheriae* (Lubowski (1900) and Gordon (1901–2)).

Summary and Conclusions.

(1) A localised epidemic of diphtheria of a clinically mild type (both as regards local and general symptoms) was associated with the prevalence in the fauces of patients and contacts of strains of *B. diphtheriae* of low pathogenicity for animals; 35 % of these strains possessed moderate virulence for guinea-pigs, 30 % low virulence and 35 % were non-virulent.

(2) Prolonged nasal discharge after moderately severe diphtheria was in one case associated with a strain of *B. diphtheriae* which was non-virulent for guinea-pigs.

(3) Acid production in various carbohydrate media proved a valuable means of differentiating diphtheria-like bacilli, but the exact composition of the medium is of importance. Peptone water appears to be especially suitable for the basis as being least liable to variation.

(4) The agglutination test gave a fairly uniform result with most of the strains of virulent *B. diphtheriae*, but three virulent strains did not give a decided positive reaction to this test. Of the non-virulent strains half gave a slight, the remainder a negative result.

Bacillus pseudo-diphtheriae (Hofmann) and certain acid producing diphtheroids gave no reaction.

(5) *B. fusiformis* and spirochaetes occurred in large numbers on the fauces in this epidemic of diphtheria, in association with the *B. diphtheriae.*

(6) After all the boys had received prophylactic injections of antitoxin and after the carriers had been isolated the epidemic promptly ceased.

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N° 11

A NOTE ON THE QUANTITIES OF MARSH-GAS, HYDROGEN AND CARBON DIOXIDE PRODUCED IN THE ALIMENTARY CANAL OF GOATS.

By A. E. BOYCOTT and G. C. C. DAMANT.

(*From the Lister Institute of Preventive Medicine.*)

It is well known that in herbivorous animals fermentation in the alimentary canal produces a not inconsiderable amount of CO_2 which in respiration experiments cannot be separated from that arising by tissue metabolism. Since these fermentations are, for the most part at any rate, anærobic, a knowledge of the quantity of CO_2 arising therefrom is of importance, not only in measuring the CO_2 production as an index of tissue activity, but also in calculating the respiratory quotient. This intestinal CO_2 cannot be measured directly: considering however that the chief fermentations—those of carbohydrates—which give off CO_2 produce at the same time hydrogen and marsh-gas, an attempt has been made to arrive at some idea of its quantity by (1) observing the total quantities of CO_2, H and CH_4 given off by animals, and (2) estimating the ratio between the CO_2 and combustible gases arising from the fermentation of the contents of the stomach and intestines.

The present series of experiments were made upon goats in an air-tight steel pressure chamber of 9500 litres capacity without ventilation. Controls showed that the production of CO_2 by the chamber itself, even when much soiled with fæces and urine, was negligible and that of H and CH_4 *nil.* Several goats were put in the chamber for each experiment. The animals were fed on hay with a small quantity of oats: with one exception the experiments began 2 or 3 hours after a meal and no food was given until the observation was completed. Hourly samples of the air inside the chamber were withdrawn and analysed in duplicate or triplicate with a delicate form of the Haldane apparatus, and in all cases the CO_2 production in the successive periods was found to be quite regular. The combustible gases were burnt with an incandescent platinum spiral and the results calculated on the assumption that no combustible gases other than CH_4 and H were present. The analytical figures were never in disagreement with this assumption, and several estimations of the oxygen consumed in the combustion were concordant with it.

(1) The results of ten experiments are shown in the following table:—

	Sex	No.	Weight kg.	Duration of exp. hours	Temp.	C.c. per kg. per hour at 0° & 760		CH_4+H =p.c. of CO_2	Resp. quotient	
						CO_2	CH_4+H			
I	M	6	111·8	3	17°	513	30·4	5·9	1·03	
II	M	6	121·3	5	13	504	20·6	4·1	1·06	
III	M	7	142·1	7	15	343	17·4	5·1	0·91	
IV	M	6	126·3	6	16	318	11·8	3·7	0·82	asting 20 hours.
V	M	7	140·9	4	15	394	14·6	3·7	—	at +21 lbs. pressure.
VI	M	7	142·7	4	13	455	26·4	5·8	—	at +45 lbs. pressure.
VII	M&F	13	295·7	4	14	344	19·6	5·7	1·08	at +45 lbs. pressure.
VIII	F	6	138·8	6	11	346	22·7	6·6	0·90	
IX	F	8	193·7	7	15	283	10·0	3·5	0·82	
X	F	8	193·7	4	13	323	18·4	5·7	—	at +45 lbs. pressure.
Average						382	19·2	5·0	—	
Average of 7 complete exps.						379	18·9	5·0	0·95	

These results show that goats produce from 10 to 30 c.c. of H and CH_4 per kilogram per hour[1] which is equal to from 3·5 to 6·6 per cent. by volume of the total CO_2 produced in the same time. On an average the combustible gases equal 5 per cent. of the total CO_2, and an average goat of 20 kilograms produces about 9 litres a day. The proportion between H and CH_4 is variable: on an average the CH_4 forms two-thirds of the total combustible gas[2].

(2) In the next series of observations the determination of the ratio between the CO_2 and the combustible gases given off by fermentation of the contents of the alimentary canal was approached in several ways. The figures given below indicate the volume ratio $\frac{CO_2}{CH_4 + H}$. The total quantity of gas arising by fermentation in different parts of the stomach

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[1] This corresponds with the results of Colasanti (*Pflüger's Arch.* XIV. p. 92, 1877), who found that guinea-pigs gave off on an average 24·6 c.c. of CH_4 and H per kilo per hour $= 2·6\,\%$ of the total CO_2. In cows the CH_4 equals about $2·8\,\%$ of the total CO_2 (Kellner, *Landwirtsch. Versuchs. Stat.* LIII. 1900). See also D. Finkler (*Pflüger's Arch.* XV. p. 603, 1877), Wolfers (*ibid.* XXXII. p. 265, 1883), and J. A. Fries (*Amer. Journ. Physiol.* XVI. p. 468, 1906).

[2] It is to be noted that the combustible gases produced in experimental fermentations of carbohydrates are variable. For the most part sugars give off H and cellulose CH_4, but cellulose may be decomposed with the production of H and there is evidence that one type of fermentation may change to the other.

and intestines cannot be exactly determined *in vivo* owing to the exchange, partly of a differential kind[1], which is going on with the blood; and results as to absolute quantities obtained by observations *in vitro* cannot be regarded as in any way reliable in detail. As far as they go, such experiments show that the greater part of the gas is given off in the first, second, and third stomachs. A considerable experience of the appearances found in freshly killed goats bears this out. The stomach habitually contains several litres of gas while the intestines never show more than a few bubbles, and though the contents of the small intestine give off a fair amount outside the body, the contents of the first and second stomachs give off far more, while from the contents of the cœcum and fourth stomach only very small quantities are obtained. In the large intestine the contents are dry and fermentation has practically ceased. It may also be noted that the contents of the stomach considerably exceed in bulk those of the rest of the alimentary canal, weighing on an average 6 times as much. It also appears that most of the gas leaves the body *per os* and not *per anum*: the actual passage may be easily observed in goats. Under a pressure of + 45 lbs. the apparent production of combustible gas in several experiments was *nil* for as long as two hours, and a rapid diminution in pressure resulted in an immediate increase: this is not in accord with the supposed exit of the greater part of the intestinal gases *via* the lungs[2].

A. Gas found in the stomach immediately after death (5 goats).

$$\frac{CO_2}{CH_4+H}=15{\cdot}2;\ 2{\cdot}0;\ 2{\cdot}3;\ 5{\cdot}4;\ 1{\cdot}8.$$

B. Stomach removed entire and incubated at 36°—37°, the gas which accumulated being sampled and emptied out from time to time. The peritoneum soon dries and the stomach becomes fairly gas-tight.

	I		II	III
0—2 hours	1·5	0—2 hours	11·3	1·35
2—5	1·7	2—4	14·6	1·7
5—9	2·1	4—6	12·8	
9—11	2·0	6—8	11·2	
11—33	3·0	8—21	10·9	
33—38	2·3			
38—41	3·1			

In experiments I and III the combustible gas was almost all CH_4: in II chiefly H.

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[1] Boycott. This *Journal*, xxxii. p. 343. 1905.

[2] Zuntz. *Arch. f. Physiol.* p. 354. 1894.

C. Contents of stomach, etc. removed and incubated *in vitro* at 37°, the gas being collected over mercury.

	1st stomach	2nd stomach	3rd stomach	4th stomach	Small int.	Cœcum
0— 2½ hrs.	7·0	—	—	—	2·1	31·0
3—20	7·6	9·1	6·8	8·0	3·6	26·1
25—27	—	4·9	—	—	—	—
27—51	15·2	5·6	36·6	31·4	22·3	—
51—75	12·7	18·4	15·3	32·2	12·7	—

In another experiment the contents of the first stomach gave ratios of 5·1, 5·6, 7·4, 9·3 and 10·2 in five successive periods of 24 hours.

The observed ratios $\frac{CO_2}{CH_4 + H}$ thus vary within very wide limits. Disregarding on the one hand the high ratios observed when the contents had been removed from the body for many hours, and considering on the other hand that the loss by escape through the stomach walls, saturation of the contents of the fermentation flasks, etc. would fall on the CO_2 rather than on the combustible gas, it would seem not unreasonable to assume that at least twice as much CO_2 as combustible gas arises from fermentation in the alimentary tract[1]. Since the combustible gas equals 5 % of the total CO_2, it follows that on an average at least 10 % of the total observed CO_2 arises from fermentation and not from tissue metabolism.

There is little doubt that the bacterial action concerned in these processes goes on anærobically. There is therefore a correction to be made in the observed respiratory quotient. On the assumption that the ratio $\frac{CO_2}{CH_4 + H} = 2$, the figures already given are corrected as follows :—

Number	$CH_4+H=$ p.c. of CO_2	Observed R. Q.	Corrected R. Q.
I	5·9	1·03	0·91
II	4·1	1·06	0·97
III	5·1	0·91	0·82
IV	3·7	0·82	0·76
VII	5·7	1·08	0·96
VIII	6·6	0·90	0·78
IX	3·5	0·82	0·76

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[1] It is possible that some of the methane and hydrogen may be used up in the alimentary canal by the bacteria recently described by Söhngen (*Cent. f. Bakt.* Abt. II. xv. p. 513, 1905), Kaserer (*ibid.* p. 573 and XVI. pp. 681 and 769, 1906), and Nabokich and Lebedeff (*ibid.* XVII. p. 350, 1906). This occurrence would increase the quantity of CO_2 arising from the intestine by the amount of CH_4 oxidised, but it would not very materially affect the present calculation.

The mean respiratory quotient falls from 0·95 to 0·85 (at most), a figure which is not in accordance with the view that cellulose is dealt with by the tissues as a carbohydrate rather than as a fatty acid arising from the carbohydrate by bacterial action before assimilation.

It would seem appropriate that the respiratory activity of different animals should, for comparative purposes, be expressed in terms of their "clean" weight, *i.e.* the total weight less the contents of the alimentary canal. A number of observations have shown that the contents of the stomach and intestines constitute on an average 18·8 °/₀ of the total body weight in goats, the extreme figures being 16·4 °/₀ and 22·7 °/₀[1]. A series of 27 estimations of the CO_2 production of normal goats of 12 to 30 kilos, including those given above, gives an average of 410 c.c. (0·811 grm.) per kilo per hour, and 997 c.c. (1·971 grms.) per 1000 sq. cm. of surface. Deducting the estimated CO_2 production of the contents of the alimentary canal and reducing the weight of the animals by the weight of these contents, we obtain a corrected mean figure of 453 c.c. (0·896 grm.). This correction would be more important with small animals having considerable intestinal contents.

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[1] Proceedings of the Physiological Society, this *Journal*, vol. xxxvi. p. xiv. 1907.

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On the Relation between the Output of Uric Acid and the Rate of Heat Production in the Body.

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(Communicated by Dr. C. J. Martin, F.R.S. Received June 22,—Read June 27, 1907.)

(From the Lister Institute of Preventive Medicine.)

The rate at which uric acid is turned out of the body is very different at different times of the day, even when the food contains no ready-made purine derivatives. It is higher during the early hours of the day than at any time, and it is considerably lower at night. The reason for this, as was pointed out by one of us,[*] is not likely to be that the excretory functions are depressed at night, since these functions, to judge from the total nitrogen of the urine, are more active during the first hours of sleep than at any time in the 24 hours. And since, when the diet is confined to bread, butter, and milk, the uric acid must be derived from the body substance and not from the food, it seems probable that there is some function of the body which is in abeyance during sleep and is, to a considerable degree, responsible for the output of uric acid; some function, that is to say, which is effected by chemical reactions involving the production of uric acid, and of nothing else that can be detected in the urine, unless it be phosphoric acid, and possibly in some measure creatinine.

If it is possible to identify this function, the activity of which can, on a suitable diet, be measured by the amount of uric acid excreted, it may be possible to give a clearer account of the processes by which, at the onset of fever, the temperature of the body can be sent up independently of any voluntary muscular activity; for while the temperature is rising, the output of uric acid may be four times as great as it otherwise would be.[†] Similarly, in the study of other pathological conditions in which uric acid plays a part, it must be of importance to be able to point to the kind of activity which is accompanied by increased uric acid production.

The following series of observations, which we have worked out conjointly, was designed to show how the reaction of the body to loss of heat on the one hand, and the heat produced by ordinary muscular exertions on the other, might affect the amount of uric acid in the urine. It was the outcome of several earlier, as yet unpublished, experiments, which pointed, though less clearly, to the same conclusions as we think may be drawn from this one.

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[*] J. B. Leathes, 'Journal of Physiology,' vol. 35, p. 125, 1906.

[†] *Loc. cit.*, p. 205.

A diet was taken by the subject of the experiment (E. P. C.) which consisted of 2 quarts of milk (2·28 L.), 12 ozs. of bread (360 grammes), 4 ozs. of cheese (120 grammes), and 2 ozs. of butter (60 grammes). This was distributed equally over six identical meals taken every three hours from 7 A.M. to 10 P.M.

The urine was collected immediately before each meal, and in each sample the total nitrogen (Kjeldahl), the creatinine (Folin), the uric acid (Folin), and the phosphoric acid (by titration with uranium acetate) was estimated, and the hourly rate of output calculated for each period of the day. The diet was taken from May 17 till June 4. On the first two days the urine was collected for 24 hours and mixed before analysis.

On the eighth day of the diet, May 24, two and three-quarter hours were spent stripped at a swimming bath. For the greater part of this time the subject of the experiment stood about, and entered the water only from time to time as the skin became dry, the object being to lose heat with as little compensatory production of heat by voluntary exertion of the muscles as possible. The rectal temperature was reduced in this way from 100°·8, at 2.50 P.M. immediately after undressing and after the 10 minutes' walk to the bath, to 97°·7 just before dressing again at 5.30. The reading at 4 P.M. was 98°·8, and at 5 P.M. 98°. The temperature of the water was 71°, and that of the air 64°. At fairly frequent intervals spasmodic attacks of shivering came on, but these were not so severe as in a "rigor." Although the body temperature fell 3° F., the greater part of this fall would have occurred even with clothing, as the observations taken every three hours throughout the whole experiment showed that a rectal temperature of 98°·8, or even slightly less, was normal for this time of day on abstaining from muscular activity. The body temperature, therefore, fell barely a degree beyond the normal range of variations, and the regulating function was not seriously overtaxed. This was the result aimed at, as a previous experiment had shown that a more severe and rapid removal of body heat introduced disturbing factors and gave a different result.

The effect on the output of uric acid was to increase it more than 80 per cent. in comparison with the mean of the four preceding days. The figures observed are given in Column C of Table I appended.

Three days later, May 27, the exposure to cold was repeated, only with this difference that active muscular exertions were introduced. The subject was stripped for two and three-quarter hours and kept his skin wet by repeated immersion in, and sponging with, cold water, T.° 59 to 60. In the intervals quick-running movements, maintained for five minutes at a time, and dumb-bell exercises, sufficient to cause pronounced hyperpnœa, were carried out. In this way actual shivering was almost entirely avoided, although the periods of immersion, longer than those on the 24th, and the sponging with water 10° colder than that at the swimming bath, the measures that is for removing body heat, were more drastic than on the former occasion. The body temperature, which was 100°·4 at 4.10 P.M. when the treatment began, was still 100° at 5.30; it was lowest (98°·8) at 6.30, but was slightly raised again (99°·1) at 6.50, when the treatment ended.

The effect on the output of uric acid, shown in Column D of Table I, was in the succeeding 24 hours to increase it, but only to about half the extent to which it was increased by the cold without muscular exertions three days before, and if the comparison be made with the output of the 24 hours immediately preceding, *i.e.*, 312 milligrammes, the increase is less than 20 per cent., and during the three hours of the treatment the output was remarkably low.

Three days later, on May 30, the subject of the experiment, dressed in winter clothing, with, in addition, a thick knitted jersey, spent the greater part of two and three-quarter hours in severe muscular exertions. At 3.10 P.M., with the rectal temperature at 99°·8, a sharp walk of 2½ miles, lasting half an hour, raised the temperature to 102°·4. Then, after an interval of a quarter of an hour for food, a heavy floor rubber was worked to and fro for half an hour, at the end of which time the temperature was 101°·8. The walking was then repeated (T.° 102·1) and finally the work with the rubber for half an hour more giving a temperature at 5.50 P.M. of 102°.

The output of uric acid fell from 311 milligrammes in the preceding 24 hours to 223 in those including and succeeding the exercise. The figures are given in Column E of Table I.

Finally, four days later, on June 4, an attempt was made to repeat the experimental conditions of the first exposure to cold on May 24 in the swimming bath. It was not possible to do this exactly, because the air temperature was 8° lower, 56° F., and at this temperature the loss of heat was sufficient to cause shivering, which was fairly continuous for two hours. The body temperature was 98°·4 at 4 P.M. as compared with 98°·8 at the same time on the earlier occasion, but at 5.30, just before dressing, it was a trifle higher than the first time, 98° instead of 97°·7. Whether these differences in the conditions account for the result shown in Column E, which is of the same character though not so pronounced as that in Column C, we are not yet prepared to say. The output in the 24 hours preceding the final exposure was 311 milligrammes, this was raised to 386 milligrammes. This is less than the output on the former occasion, but, as on that occasion, the figures for each of the periods in the 24 hours are above the average and particularly that for the period ending at 7 P.M., which included the greater part of the exposure to cold.

On May 19, the third day of the diet, the body weight was 70·5 kilogrammes; on June 4 it had risen to 73·2 kilogrammes.

The results of this experiment point to the inference that one of the factors largely concerned with the production of uric acid in the body and its appearance in the urine is the reaction of the body to loss of heat; and that a part, it may be a large part, of the endogenous uric acid is, so to speak, the chemical expression for the intensity of this reaction; further, that this reaction involves some form of activity distinct from voluntary movements of the skeletal muscles, since such voluntary movements have, on the contrary, the effect of diminishing the output of uric acid, at any rate while they are going on.

At the same time we are not prepared to say that the activity for which the chemical expression is the output of uric acid, has no other physiological expression than heat production in response to increased loss of heat. That, indeed, would not at all represent our view. For it is at any rate clear, from this experiment taken by itself, that the reaction set up by exposure to cold may last on for a considerable time after the exposure. A glance at the column in Table II, in which the output of uric acid for each 24 hours throughout the experiment is shown, makes it obvious that the interval of three days that elapsed between each abnormal phase of the experiment was not sufficient to allow a complete return of the rate of output to its value in the days before the first exposure.

The nature of the activity concerned in this reaction to cold, and certain other points arising out of the data in Table II, will be entered into more fully on another occasion.

Table I.—Showing the Rate of Output of Uric Acid, in milligrammes per hour, for the different periods of the day ending at the hours indicated in the first column.

	A. Mean of all the 16 days.	B. Mean of four normal days before exposure to cold.	C. First exposure to cold, without exercise, from 2.50 to 5.30 P.M.	D. Cold + exercise, from 4.10 to 6.50 P.M.	E. Exercise, warmly dressed, from 3.10 to 5.50 P.M.	F. Second exposure to cold, without exercise, from 2.50 to 5.30 P.M.
7 P.M.	13 ·1	11 ·5	19 ·5	6 ·2	3 ·6*	17 ·7
10 ,,	14 ·7	13 ·0	21 ·1	20 ·4	6 ·9	15 ·5
7 A.M.	10 ·9	9 ·2	17 ·1	12 ·4	9 ·2	14 ·7
10 ,,	18 ·6	15 ·3	28 ·7	20 ·7	13 ·8	21 ·6
1 P.M.	14 ·5	12 ·0	20 ·2	19 ·2	11 ·0	14 ·7
4 ,,	13 ·9	11 ·5	17 ·4	19 ·6	11 ·3	14 ·9
Total for 24 hrs.	321 ·0	259 ·0	475 ·0	369 ·0	223 ·0	386 ·0

* This figure is almost certainly too high.

Table II.—Total Daily Output of the various Urinary Constituents estimated.

Date, 24 hours ending 4 P.M.	Uric acid.	Total nitrogen.	Creatinine.	P_2O_5.	H_2O.	Air temperature.		Remarks.
						6 P.M.	Noon.	
	mgms.	grammes.	grammes.	grammes.	c.c.	°	°	
May 20	335	16 ·25	1 ·50	3 ·27	1260	49	47	
,, 21	280	17 ·37	1 ·49	3 ·69	1582	45	51	
,, 22	225	18 ·22	1 ·46	3 ·77	1544	52	53	
,, 23	259	17 ·40	1 ·51	3 ·74	1378	59	65	
,, 24	270	17 ·73	1 ·49	3 ·35	1314	67	65	
,, 25	**475**	18 ·81	1 ·44	4 ·04	1083	64	70	2¾ hours stripped at swimming bath at beginning of this period
,, 26	338	18 ·74	1 ·51	3 ·83	1295	67	57	
,, 27	312	17 ·57	1 ·48	3 ·64	1429	58	61	
,, 28	**369**	17 ·88	1 ·51	3 ·33	1295	66	57	2¾ hours' exercise stripped and cold
,, 29	314	17 ·59	1 ·49	4 ·20	1616	51	54	
,, 30	311	17 ·07	1 ·49	3 ·93	1578	52	54	
,, 31	**223**	17 ·98	1 ·57	3 ·65	1002	57	64	2¾ hours' exercise heavily dressed
June 1	375	18 ·62	1 ·56	4 ·19	984	61	64	
,, 2	354	18 ·95	1 ·54	4 ·06	1266	59	58	
,, 3	309	19 ·39	1 ·46	4 ·24	1625	54	54	
,, 4	**386**	20 ·11	1 ·43	4 ·12	1608	56	—	2¾ hours stripped at swimming bath
Mean of 16 days	321	18 ·10	1 ·49	3 ·89				

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AN INVESTIGATION OF THE LAWS OF DISINFECTION.

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Introduction.

The work of Pasteur, showing the action of preservatives to be due to their toxic effect upon micro-organisms, and the extension of the same explanation, by the work of Lister, to cover the case of disinfectants and disease germs, was followed by a considerable amount of successful work dealing with the disinfectant action of vast numbers of substances upon putrefying matter. (Buchholtz 1875 ; Jalan de la Croix 1881.)

Robert Koch (1886), however, is responsible for the first systematic experiments on disinfection, using pure cultures of bacteria. By means of his "thread" method he investigated the effect on anthrax spores of the then popular disinfectants, carbolic acid and sulphur dioxide, and of many substances previously uninvestigated. He confirmed previous work, demonstrating the powerful disinfectant, and even greater antiseptic, properties of mercuric chloride, and the great reputation of this salt may be said to date from these experiments.

Shortly afterwards much interest was excited by the appearance of the first of many emulsified disinfectants, creolin, and this substance was investigated by many contributors to the study of disinfection, *e.g.*, Esmarch (1887) working with putrefying liquids, and Henle (1889), who worked with cholera and typhoid. The latter also showed that creolin contained higher homologues of phenol (cresols, etc.) which, themselves only slightly soluble in water, are conveniently emulsified by the addition of soap; these higher phenols are at the same time more powerful disinfectants and less poisonous than carbolic acid. Fraenkel (1889) showed that the extra disinfecting power of crude over pure carbolic acid was due also to the admixture of higher phenols; he investigated these compounds in pure samples and demonstrated their excellent disinfecting properties by means of experiments with anthrax spores.

Geppert (1889 and 1891) published experiments which showed that the disinfecting powers of mercuric chloride had been over-estimated, and criticised previous work and especially Koch's "thread" method, in view of the unavoidable carrying-over of traces of sublimate from the disinfecting fluid into the test cultures, growth being thus inhibited, although the organisms might not have been destroyed at the time of sub-culture. He proved this by adding to his test culture enough ammonium sulphide to precipitate the mercuric chloride carried over, and he worked directly with emulsions of bacteria in place of the silk threads soaked in broth cultures.

Behring (1890) published the results of a number of experiments upon disinfection and vigorously combated Geppert's views in respect of mercuric chloride, but Koch's "thread" method has since been largely abandoned for the above reason.

In 1897 Krönig and Paul published their classic work upon disinfection; their "garnet" method will be referred to later, and by its means they studied most carefully the disinfecting process, using definite species of organisms and disinfectants of every class in widely varying concentration. Their experiments were conducted with the utmost care, and the valuable quantitative results which they obtained form the most important contribution to the subject which has yet appeared.

The more recent work on disinfection has been concerned chiefly with the standardisation and comparison of different disinfectants from a practical point of view. Rideal and Walker (1903) described a method by means of which the value of disinfectants could be expressed as a numerical ratio, using pure phenol as a standard. Their method, with certain modifications, was used in the present investigation and was briefly as follows:—a definite small amount of broth cultures of the same species and age was added to a constant volume of disinfectant solution. By making a series of trials with varying concentrations of the disinfectant to be tested, and of pure phenol under otherwise similar conditions, they determined the relative concentrations of the two fluids required to bring germicidal action to completion in the same time. The ratio of the reciprocals of such concentrations was taken to express the disinfecting value of the unknown disinfectant in terms of carbolic acid as a standard. This ratio was called by them the 'carbolic acid coefficient.'

Madsen and Nyman (1907), using Krönig and Paul's method, have further investigated the progress of the reaction during the disinfection of anthrax spores by mercuric chloride and heat. Their experiments are in many ways parallel to my own and their results will be discussed later.

Three classes of disinfectants have been investigated in the present research.

(1) Dissolved organic disinfectant:—phenol.

(2) Emulsified disinfectants, containing insoluble coal tar derivatives held in very fine suspension in water by means of soap, glue, etc.:—disinfectant "A."

(3) Metallic salts:—mercuric chloride and silver nitrate.

SECTION I. REACTION VELOCITY OF DISINFECTION.

Krönig and Paul (1897) published the results of a few careful experiments with anthrax spores, using mercuric chloride of different concentrations. Their method was as follows:—garnets were selected of equal size, carefully cleaned and dipped into an emulsion of sporing anthrax bacilli, which were allowed to dry on their surface in a thin film. The garnets were immersed in the solution of mercuric chloride, and from time to time a definite number was taken, and all traces of sublimate were removed by gentle washing and treatment with ammonium sulphide. These garnets were then well shaken in a measured quantity of water to detach the adherent spores and a constant amount of the washings was plated. The number of germinating organisms was counted. It was shown that by thus shaking in water a fairly constant definite proportion of the total organisms was detached.

From a study of the figures obtained in Krönig and Paul's experiments, it is evident that the disinfection process is a gradual one. No definite conclusions were drawn by the authors themselves, but Ikéda (1897) attempted to explain the course of the reaction and deduced from their results the following formula:—

$$\frac{n_1}{n_2} \times \frac{t_1}{t_2} = \text{constant},$$

where n_1 and n_2 are the numbers of organisms surviving after times t_1 and t_2 respectively.

On plotting the results of Krönig and Paul (see Fig. 1, ordinates representing the numbers of surviving bacteria, and abscissae the corresponding times), the points were found to lie upon fairly smooth curves. The form of these curves suggested the existence of a logarithmic relation between the two variables, time on the one hand, and the number of surviving bacteria on the other. In fact the curves appeared to be very similar in form to that expressing the course of a so-called "unimolecular reaction," and the equation

$$-\frac{dC}{dt} = KC,$$

or

$$\frac{1}{t_2 - t_1} \log \frac{C_1}{C_2} = K$$

was found to be applicable to the case of disinfection, if, in place of the terms C_1 and C_2 expressing concentration of reacting substance, numbers of surviving bacteria were inserted, thus:—

$$\frac{1}{t_2 - t_1} \log \frac{n_1}{n_2} = K,$$

where n_1 and n_2 are the numbers of bacteria surviving after times t_1 and t_2 respectively.

In their recent work Madsen and Nyman (1907) also discover from Krönig and Paul's figures that, in the disinfection of anthrax spores with mercuric chloride, the reaction proceeds according to the equation given above. A table[1] is given (p. 390) showing the good agreement between the numbers of surviving bacteria actually counted by Krönig and Paul, and those calculated by Madsen and Nyman on the supposition that the above relation holds good. Their own experiments with mercuric chloride and anthrax spores, using Krönig and Paul's methods, have fully confirmed the previous work and have shown that the same law holds good for the destruction of anthrax spores by heat.

It was necessary, first of all, to repeat[2] these enumeration experiments of Krönig and Paul, using a different germicide, and the following experiments were therefore made with anthrax spores, phenol being substituted for mercuric chloride. In order to obtain perfect comparison with further experiments upon a non-sporing organism (*Bacillus paratyphosus*), for which the garnet method was unsuitable, the experiments were made as follows:

An agar culture of anthrax, about a week old, consisting almost entirely of spores, was emulsified with a little distilled water and centrifugalised for a few minutes to get rid of any scraps of agar or aggregated masses of spores. The supernatant liquid was pipetted off and heated to 80° C. for about five minutes. This formed the emulsion used for the experiments ; it was preserved in the cold room and was found to suffer very little change.

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[1] This table will be referred to again later (Section II, p. 130).

[2] Some enumeration experiments of Spiro and Bruns (1898) with pyrocatechin and anthrax spores roughly confirm those of Krönig and Paul; they were, however, not made with any very great accuracy, but form an interesting confirmation.

Everything used in the experiments, tubes, pipettes, etc., was previously sterilised, and the disinfectant, in this case phenol (5 %), was measured into a test tube and placed in a water-bath whose temperature was maintained constant, in most experiments at 20° C. A small amount of the emulsion (depending upon the concentration of spores, determined by a previous control experiment) was added to the disinfectant tube. At definite intervals of time samples were withdrawn, diluted, and immediately plated on agar. These samples were obtained by means of a capillary pipette fitted with a rubber cap and fixed by means of a cotton wool plug into the mouth of the disinfectant tube. These pipettes were specially made so that the external diameter at the mouth was constant in size ; it was then found that the size of the drop yielded was constant also, in the present instance = 0·02 c.c.

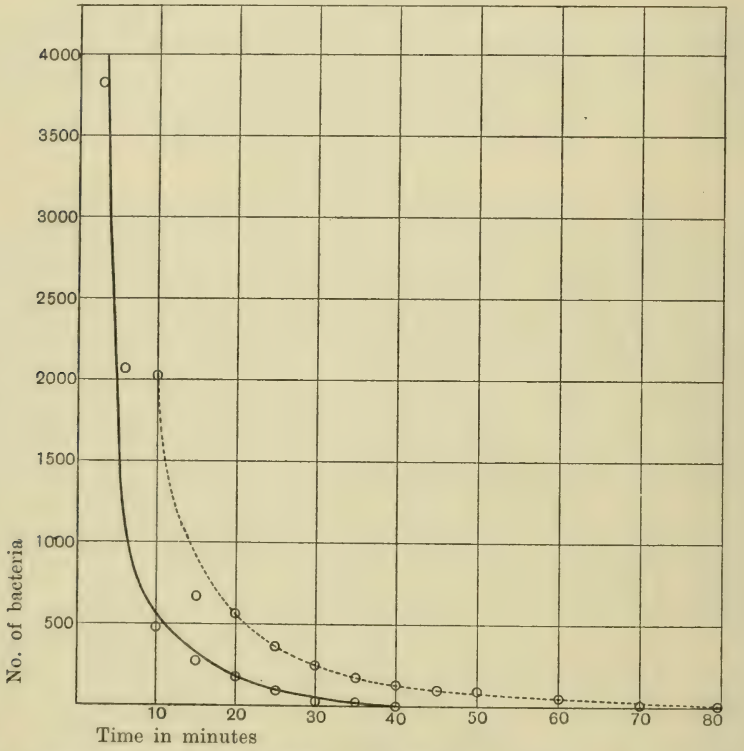

Fig. 1. Illustrating the results of Krönig and Paul's experiments. Continuous curve, disinfection of anthrax spores with 2·1 per 1000 $HgCl_2$. Dotted curve, disinfection of anthrax spores with 1·1 per 1000 $HgCl_2$.

A disadvantage of this method, compared with the "garnet" method, is that the trace of disinfectant adhering to the sample is incubated along with it. In the "garnet" method these traces are removed by washing before incubation. However, in the case of phenol, this drawback was found to be unimportant, owing to the great dilution the sample underwent. At the beginning of the experiments the samples were one drop (= 0·02 c.c.); later, when the number of survivors was very much reduced, they were increased to 4 or 6 drops. Although in the case of anthrax spores the disinfectant, phenol, was very concentrated (5 %), in the sub-culture plates the concentration was reduced to from 1 in 10,000 to 5 in 10,000, a concentration exerting no inhibition upon the development of anthrax spores[1].

TABLE I. (*Exp.* 15. 6. 07.)

Anthrax spores. 5 % phenol. 20·2° C.

Sample No.	Amount of sample	Time elapsing hours	Nos. of bacteria counted (6 plates)	Mean	Mean no. of bacteria present in 1 drop disinfecting mixture	K*, assuming reaction to be in accordance with the equation $-\frac{dn}{dt}=Kn$ $\left(K=\frac{1}{t_2-t_1}\log\frac{n_1}{n_2}\right)$
1	1 drop	0	441, 491, 489 365, 382	434	434, taken as initial value of $n\ (=n_1)$ in calculating values of K	
2	1	0·5	429, 340, 361 462, 443, 426	410	410	0·049
3	1	1·5	276, 398, 360 351, 341, 379	351	351	0·061
4	1	2·7	310, 322, 338 351, 287, 378	331	331	0·044
5	1	3·92	324, 280, 294 277, 289, 329	299	299	0·041
6	2 drops	5·95	528, 501, 441 495, 451	483	241	0·043
7	4	25·6	61, 77, 149 127, 135, 125	112	28	0·046

Mean value of K, 0·047.

* In Tables I—XV, values of K from expression $\frac{1}{t_2-t_1}\log\frac{n_1}{n_2}$ were calculated with Briggs' logarithms in place of natural logarithms.

In the case of anthrax it was found necessary to use as small an amount of agar as was convenient (viz. 8 c.c.), so that, after pouring, the film of agar in the petri dish formed as thin a layer as possible, and abundant oxygen was provided for the germination of the spores. The melted agar was poured at a temperature of about 65° C.; this was done to obtain perfect mixing and separation of the spores in the drop sample. For the same reason, the plates were previously warmed to about 40° C., and 0·5 to 1 c.c. sterile distilled water placed near the middle of each; the sample from the disinfectant tube was dropped into the water, and this instant was considered the exact moment of sampling. The sample was well stirred with a platinum needle, the agar then poured, and the whole well mixed. The correct enumeration of anthrax spores presents some trouble owing to difficulty in separation, and very many failures resulted before the above exact procedure was evolved and adhered to.

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[1] In the experiments with *B. paratyphosus* the concentration of disinfectant in the sub-cultures was quite insignificant, *e.g.*, phenol 1 in 200,000 to 1 in 50,000, according to the size of the sample taken. When mercuric chloride was employed it had to be neutralised.

Six plates were poured of each sample, they were incubated for 24—48 hours at 37° C., and the mean of the six enumerations taken as the result.

The results of two experiments are given in Tables I and II, the values of "*K*" are calculated, as before, from the mean values of the numbers counted on the plates; the constancy of the value is exceedingly well marked.

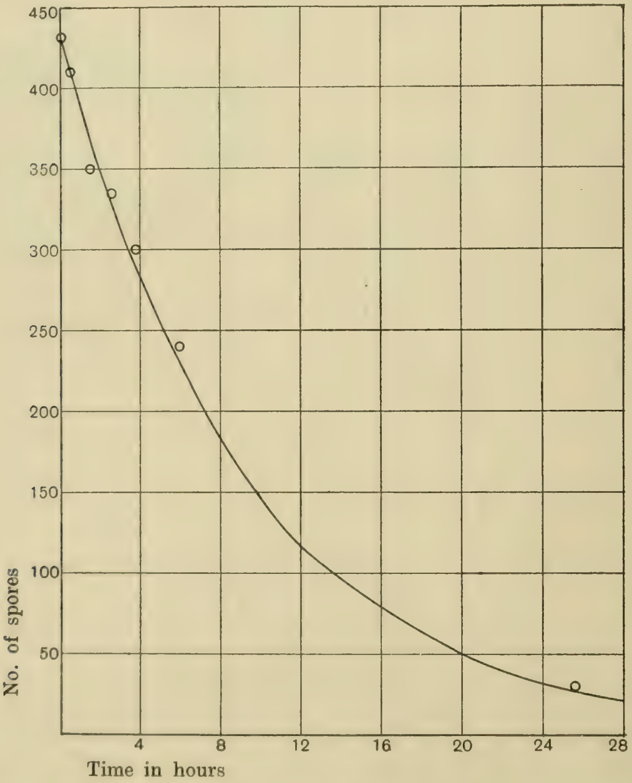

Fig. 2. (Exp. 15. 6. 07.) Showing course of disinfection of anthrax spores with 5 % phenol at 20·2° C. (see Table I). The curve is drawn through a series of points found by calculation; the circles show the results of experimental determinations.

The close agreement of the results of these two experiments with the theory is also shown in Figures 2 and 3. In the experiment at 20·2° C. (Table I), the mean value of *K* was found to be 0·047. A curve

TABLE II. (*Exp.* 18. 6. 07.)

Anthrax spores. 5 % phenol. 33·3° C.

Sample No.	Amount of sample	Time elapsing hours	Nos. of bacteria counted, mean of 6 plates	Mean no. of bacteria present in 1 drop disinfecting mixture	K, assuming reaction to be in accordance with the equation $-\frac{dn}{dt}=Kn$ $\left(K=\frac{1}{t_2-t_1}\log\frac{n_1}{n_2}\right)$
1	1 drop	0	439	439, taken as initial value of n $(=n_1)$ in calculating values of K	
2	1	0·5	275·5	275·5	0·40
3	1	1·25	137·5	137·5	0·40
4	1	2	46	46	0·49
5	2 drops	3	31·6	15·8	0·48
6	2	4·1	10·9	5·45	0·46
7	4	5	13·9	3·6	0·41
8	6	7	3	0·5	0·42

Mean value of K, 0·44.

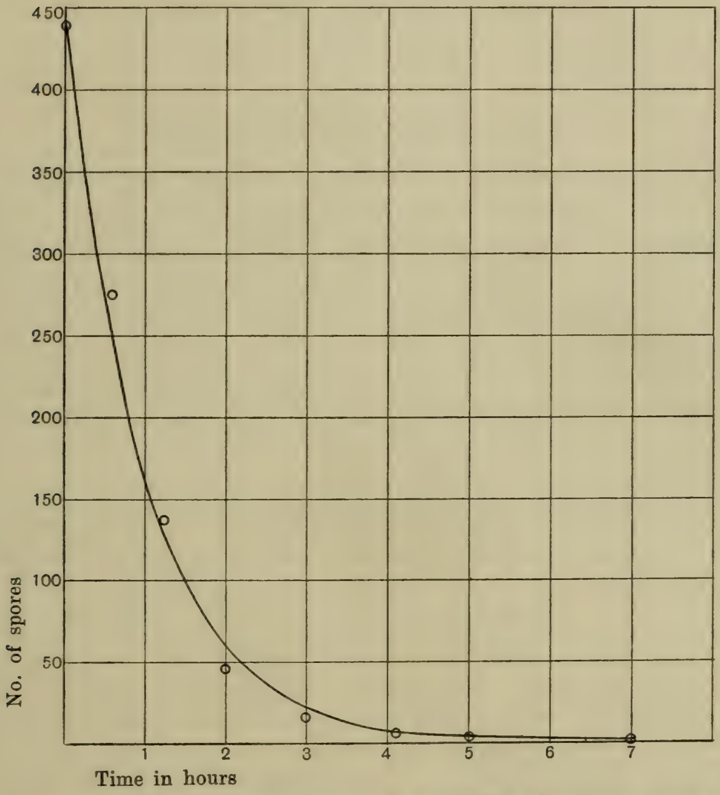

Fig. 3. (Exp. 18. 6. 07.) Showing course of disinfection of anthrax spores with 5 % phenol at 33·3° C. (see Table II). The curve is drawn through a series of calculated points, the circles show the results of experimental determinations.

(Fig. 2) was then drawn through a series of points obtained by putting $K = 0{\cdot}047$ in the equation given on p. 95, thus:—

$$0{\cdot}047 = \frac{1}{t_2 - t_1} \log \frac{n_1}{n_2},$$

n_1, the initial number of organisms, being 434. The circles, Fig. 2, represent the points actually found by experiment. The curve in Fig. 3 was drawn in a similar way using the mean value of K obtained in the experiment at 33·3° C. There is an equally close agreement between the observed and calculated points in this instance also. One may therefore rely upon this method to give as consistent results as the "garnet" method.

In the case of anthrax spores the course of disinfection, either with mercuric chloride or phenol[1], proceeds in a perfectly orderly manner and can be represented by the equation:—

$$\frac{1}{t_2 - t_1} \log \frac{n_1}{n_2} = K.$$

Experiments with B. paratyphosus.

To determine whether the law thus obtained for disinfection of anthrax spores with two different disinfectants was capable of wider application, experiments were made with a non-sporing organism. *B. paratyphosus* was chosen for most experiments because its recognition is at all times facilitated by its property of fermenting dulcite and glucose.

The disinfectants chosen were: (1) phenol, (2) disinfectant "A" (an emulsified preparation of higher coal tar derivatives), (3) mercuric chloride.

A 24 hours' broth culture was used, and in the earlier experiments this broth culture was used directly without dilution, and the samples from the disinfection tubes diluted before plating (Tables III, IV and XI).

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[1] Phenol is a weak and uncertain germicide for spores and it was found necessary to use it as strong as possible, viz. 5 %; even then the reaction at 20° C. was not complete after 28 hours. At 33° C. the disinfection proceeded much more rapidly and was nearing completion after 7—8 hours. These results roughly confirm those of previous workers using widely different methods: Koch (1886) showed phenol to be an untrustworthy disinfectant for anthrax spores, Fraenkel (1889) found them to be killed only in 40 days by 5 % phenol, while Behring (1890) found that although at room temperature many days were necessary, at 37·5° C. disinfection was complete in 3 hours. These three workers all used the "thread" method. Krönig and Paul (1897), with the "garnet" method, showed the reaction with 5 % phenol to be incomplete in 24 hours.

Dilution tubes, containing a suitable amount of sterile water, were prepared, and small amounts (1, 2 or 5 drops) added to them from the disinfection tubes by means of the capillary pipettes. From the dilution tubes, small quantities measured with capillary pipettes were plated. At the beginning of an experiment dilution was necessarily large, later on it became possible to plate samples from the disinfection tube undiluted. This method, which is very troublesome in practice and needs many trial experiments before a successful one is obtained, has the additional drawback that, whereas the object of the experiment is to obtain a series of perfectly comparable results, hardly any two of them are obtained by means of an entirely comparable procedure. There appears to be a very considerable error introduced with dilution and the use of capillary pipettes, probably due to a tendency for the suspended bacteria to be attracted to the walls of the pipettes, etc., consequently the drops yielded may not be true samples. However, if the same sampling pipette is always used, and no subsequent dilution takes place, the results are found to be fairly comparable. Later on therefore, the method was changed, the broth culture was extensively diluted before use, and a comparatively small number of organisms was placed in the disinfection tubes, less than a thousand per drop. The sample drops, all removed with the same pipette, were plated undiluted, and the plates, poured from time to time, being perfectly comparable, the results presented much greater consistency.

As has been said before (p. 97), in the case of phenol and disinfectant "A," it was not found necessary to neutralise the trace of disinfectant carried over with the sample into the test cultures, as separate experiments showed the proportion there present to exercise no inhibition upon growth. The sample drop or drops, from disinfection tube or dilution tube, were dropped upon the plate, and melted agar, about 12 c.c. at a temperature not exceeding 45° C., was immediately poured upon the plate, and the whole well mixed. As a rule duplicate plates were poured at the same instant, and this necessitated the help of one or more assistants.

In the case of mercuric chloride, the trace carried over in the sample required precipitation (see p. 123), for mercuric chloride in exceedingly small amount can effectually inhibit subsequent growth even when the organisms may have retained their vitality at the moment of sampling. Accordingly the samples were neutralised with ammonium sulphide, of which a sufficient amount was added to the dilution tube, when diluted samples were plated, or directly placed upon the plate when drops from the disinfection tube were plated undiluted. In the latter case the sample drop or drops were dropped directly into the ammonium sulphide solution and melted agar immediately poured into the plate and the whole well mixed. The results of these enumeration experiments with all three disinfectants are given in Tables III—X; phenol, Tables III—VII; disinfectant "A," Tables VIII and IX; mercuric chloride, Table X.

The values of K given by the equation

$$K = \frac{1}{t_2 - t_1} \log \frac{n_1}{n_2}$$

are also given, and it will be seen that the value of K does not remain constant as was the case with anthrax spores, but continuously decreases as the experiment proceeds. This continuous fall in the value of K occurred in the case of all three types of disinfectants worked with.

TABLE III. (*Exp.* 6. 2. 07.)

B. parat. 24 hours' culture. Phenol, 6 per 1000. 20° C.

Time minutes	Mean no. of bacteria present in diluted samples plated		Mean no. of bacteria present in 1 drop original disinfection mixture	Values of K, assuming reaction to be in accordance with the equation		
				(a) $-\frac{dn}{dt}=Kn$	(b) $-\frac{dn}{dt}=Kn^{1\cdot5}$	(c) $-\frac{dn}{dt}=Kn^2$
·5	121	× 125	15,000, taken as initial value of n $(=n_1)$ in calculating values of K			
1	110	× 50	5,500	—	—	—
2	238	× 25	5,950	—	—	—
3	41	× 25	1,025	0·27	0·009	0·00015
4	173	× 10	1,730	0·27	0·012	0·00024
5	86	× 10	860	0·26	0·012	0·00031
6	140	× 4	560	0·22	0·011	0·00038
7	131	× 4	524	0·22	0·013	0·00042
8	78	× 4	312	0·20	0·012	0·00024
9	76	× 4	304	0·18	0·011	0·00037
10	69	× 4	276	0·15	0·013	0·00056
15	25·5	× 4	102	0·12	0·011	0·00071
20	18	× 4	72	0·08	0·007	0·00048
30	—	—	71	0·06	0·007	0·00050

TABLE IV. (*Exp.* 1. 2. 07.)

B. parat. 24 hours' culture. Phenol, 6 per 1000. 20° C.

Time minutes	Mean no. of bacteria present in diluted samples		Mean no. of bacteria present in 1 drop disinfection mixture	*K*, assuming the reaction to be in accordance with the equation		
				(*a*) $-\frac{dn}{dt}=Kn$	(*b*) $-\frac{dn}{dt}=Kn^{1\cdot5}$	(*c*) $-\frac{dn}{dt}=Kn^2$
1	54	×50	2,700, taken as initial value of n $(=n_1)$ in calculating values of K			
2	12·5	×50	625	0·63	0·042	0·0012
3	6	×25	150	0·61	0·062	0·0031
4	5·5	×25	137	0·42	0·044	0·0025
5	11·5	×10	115	0·34	0·036	0·0021
7	5·5	×10	55	0·25	0·038	0·017

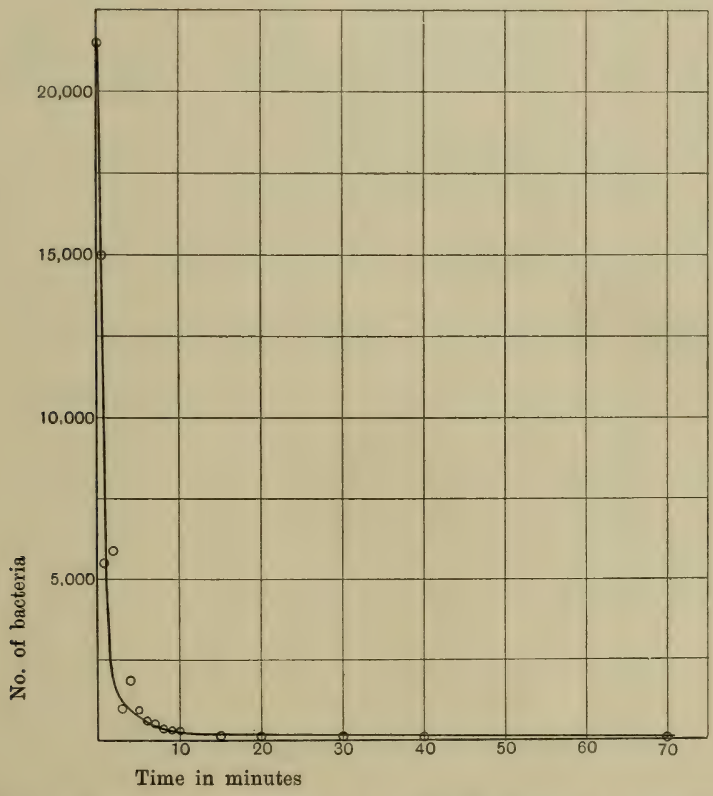
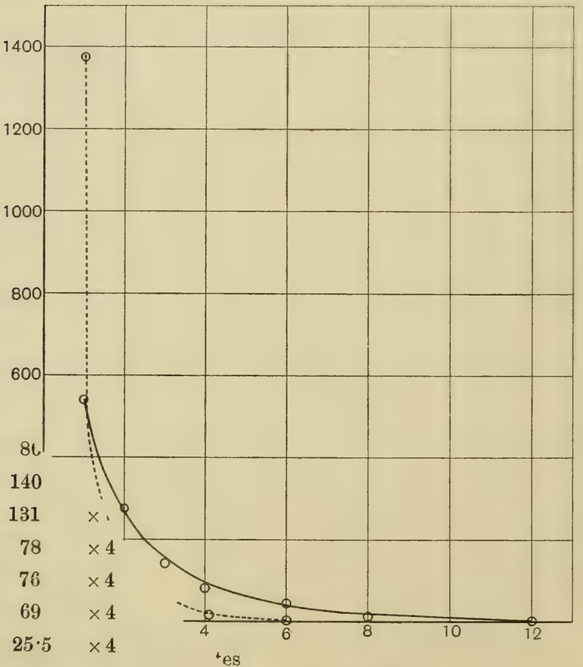


Fig. 4. (Exp. 6. 2. 07.) Disinfection of a 24 hours' culture of *B. paratyphosus* with phenol, 6 per 1000, at 20° C. (see Table III).

TABLE V. (*Exp.* 11. 7. 07.)

B. parat. 24 hours' culture. Phenol, 6 per 1000.

		Time mins.	Amount of sample taken	Nos. counted on plates	Mean	Mean no. of bacteria present in 1 drop disinfection mixture	K, assuming reaction to be in accordance with the equation $-\frac{dn}{dt}=Kn$
(*a*)	20° C.	1	1 drop	569, 509	539	539, taken as initial value of n ($=n_1$) in calculating values of K	
		2	1	357, 247, 226	276·6	276·6	0·29
		3	1	151, 124	137·5	137·5	0·30
		4	2 drops	140, 167, 174	160·3	80·1	0·28
		6	3	139, 113	126	42	0·22
		8	5	83, 90, 93	88·8	17·8	0·21
		12	7	21, 14, 12	15·7	2·2	0·22
(*b*)	30° C.	1	1 drop	1252, 1480	1368	1368, taken as initial value of n	
		2	3 drops	473, 474, 509	485	162	0·93
		3	6	438, 352, 388	392·6	65·5	0·66
		4·1	6	117, 82, 73	90·6	15·1	0·63
		6	10	8, 13, 25	15·3	1·5	0·59



4		
5	8t	
6	140	
7	131	×
8	78	× 4
9	76	× 4
10	69	× 4
15	25·5	× 4
20	18	× 4
30	—	—

72 n of a 24 hours' culture of *B. paratyphosus* with
71 ıs curve: at 30° C., dotted curve (see Table V).

TABLE VI. (*Exp.* 24. 7. 07.)

B. parat. 24 hours' culture. Phenol, 6 per 1000.

		Time minutes	Mean no. of bacteria present in 1 drop disinfection mixture	K, assuming reaction to be in accordance with the equation $-\frac{dn}{dt}=Kn$
(*a*)	11° C.	0	823, taken as initial value of n $(=n_1)$ in calculating values of K	
		1·3	202	0·47
		2·3	80	0·44
		3·3	44·7	0·38
		5·3	14·3	0·33
		7·3	4·35	0·31
		10·3	1·0	0·28
		15·3	0·22	0·23
(*b*)	21° C.	0	974, taken as initial value of n	
		1·2	73	0·94
		2·2	16·3	0·81
		3·3	4	0·72
		4·3	0	—

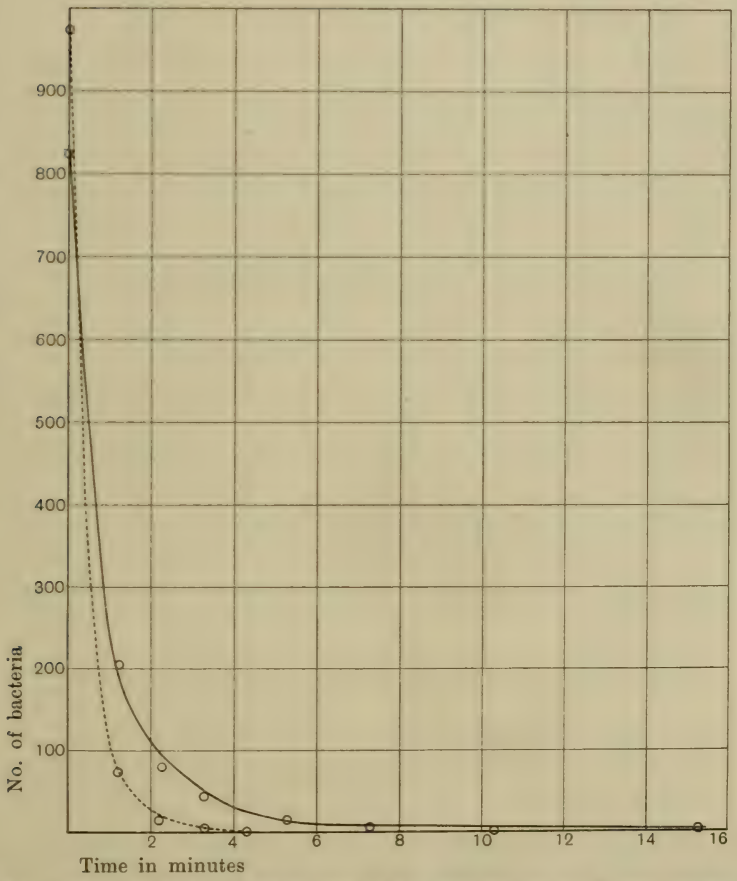


Fig. 6. (Exp. 24. 7. 07.) Disinfection of a 24 hours' culture of *B. paratyphosus* with phenol, 6 per 1000, at 11° C., continuous curve: at 21° C., dotted curve (see Table VI).

TABLE VII. (*Exp.* 23. 7. 07.)

B. parat. 24 hours' culture. Phenol, 6 per 1000.

	Time minutes	Mean no. of bacteria present in 1 drop disinfection mixture	K, assuming reaction to be in accordance with the equation $-\frac{dn}{dt}=Kn$
(*a*) 11° C.	1	765, taken as initial value of n $(=n_1)$ in calculating values of K	
	3	496	0·094
	5	271	0·112
	10	92·5	0·102
	20	5	0·062
	44	0·7	0·076
(*b*) 21° C.	1	484, taken as initial value of n	
	3	90	0·36
	5	28·5	0·31
	7	9·3	0·28
	10	3·5	0·24
	20·5	0·15	0·18

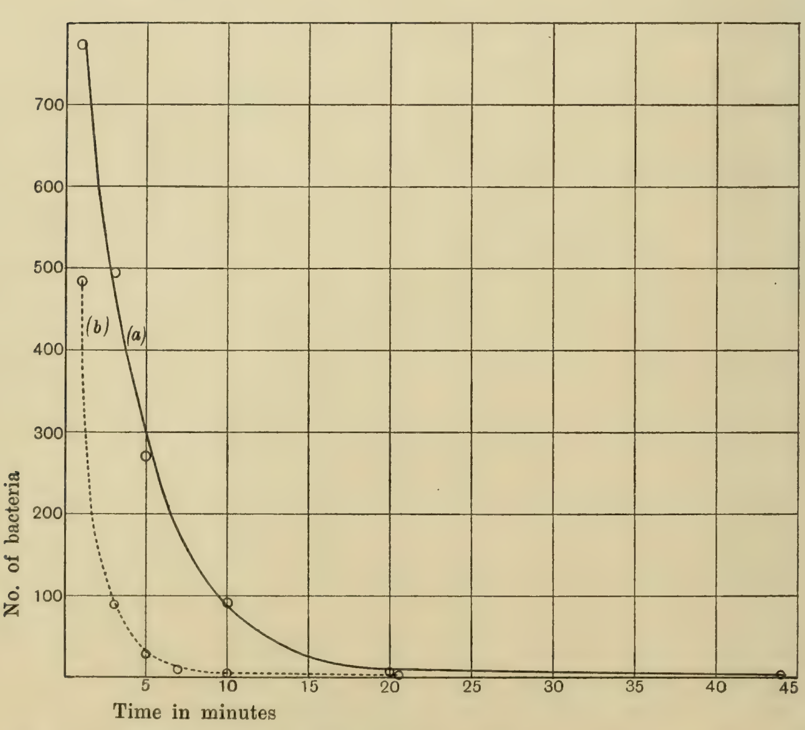


Fig. 7. (Exp. 23. 7. 07.) Disinfection of a 24 hours' culture of *B. paratyphosus* with phenol, 6 per 1000, at 11° C., continuous curve: at 21° C., dotted curve (see Table VII).

TABLE VIII. (*Exp.* 20. 3. 07.)

B. parat. Disinfectant "A," 5·5 per 10,000. 20° C.

Time minutes	Mean no. of bacteria present in 1 drop disinfection mixture	*K*, assuming reaction to be in accordance with the equations			
		(*a*) $-\frac{dn}{dt}=Kn$	(*b*) $-\frac{dn}{dt}=Kn^{1\cdot5}$	(*c*) $-\frac{dn}{dt}=Kn^{1\cdot7}$	(*d*) $-\frac{dn}{dt}=Kn^2$
0·5	370, taken as initial value of n ($=n_1$) in calculating values of K				
1	85	1·28	0·34	0·081	0·018
2	38·8	0·65	0·15	0·059	0·016
3	15	0·56	0·16	0·077	0·026
4	11	0·44	0·14	0·070	0·025
5·5	8	0·33	0·12	0·061	0·024
7	4	0·30	0·14	0·074	0·038

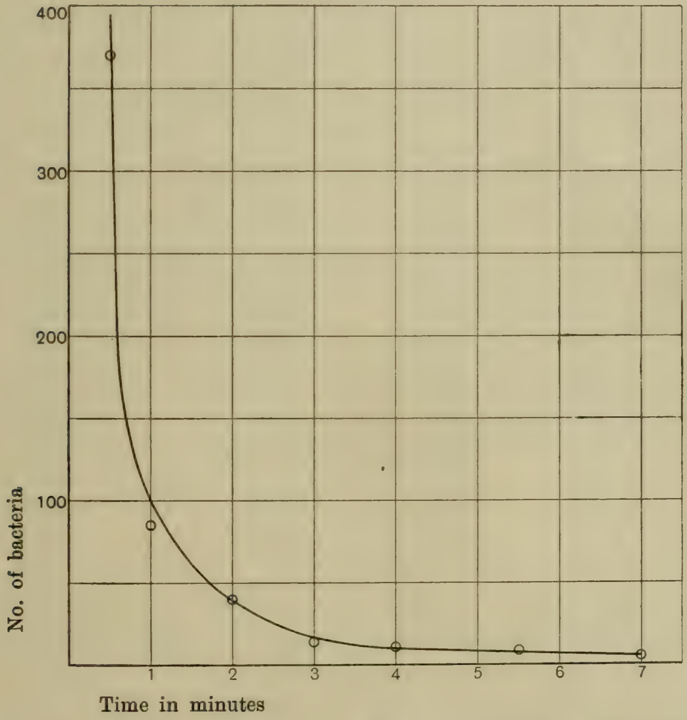


Fig. 8. (Exp. 20. 3. 07.) Disinfection of a 24 hours' culture of *B. paratyphosus* with disinfectant "A," 5·5 per 10,000, at 20° C. (see Table VIII).

TABLE IX. (*Exp.* 26. 3. 07.)

B. parat. Disinfectant "A," 5·5 per 10,000. 20° C.

Time minutes	No. of bacteria present in 1 drop disinfection mixture	K, assuming reaction to be in accordance with the equation $-\frac{dn}{dt}=Kn$
0	25,250, taken as initial value of n $(=n_1)$ in calculating values of K	
0·5	540	3·74
1	305	1·92
2·1	97	1·15
3·1	50	0·87
4·1	24	0·74
5·2	10	0·65
7	2	0·57

TABLE X. (*Exp.* 23. 3. 07.)

B. parat. Mercuric chloride, 2 per million. 20° C.

Time minutes	Mean no. of bacteria present in diluted sample		Mean no. of bacteria present in 1 drop disinfection mixture	K, assuming reaction to be in accordance with the equation $-\frac{dn}{dt}=Kn$
1·1	85	× 250	21,250, taken as initial value of n $(=n_1)$ in calculating values of K	
2·1	82	× 250	20,500	*
3	68	× 250	17,000	*
4	52	× 250	13,000	0·074
5	53	× 250	13,250	0·050
7	62	× 250	15,500	0·023
10	97	× 125	12,125	0·027
15	96	× 125	12,000	0·017
20	69	× 100	6,900	0·026
30	139	× 50	6,950	0·018
42	305	× 25	7,625	0·011
51	279	× 25	6,975	0·010
60	276	× 10	2,760	0·015
80	215	× 10	2,150	0·012
93	130	× 10	1,300	0·013
140	—	—	150	0·015
180	—	—	49	0·015
210	—	—	29	0·013
245	—	—	29	0·012

* The values of n at these times are not sufficiently accurate to give consistent values for K.

The following hypotheses are advanced to account for the facts recorded above, viz. that in the case of anthrax spores, disinfection proceeds in accordance with the equation $-\frac{dn}{dt} = Kn$, while in the case of *B. paratyphosus* this equation cannot be applied.

Let us assume that during disinfection the germicide operates upon the bacterium by so altering the constitution of its protoplasm (by chemical combination or otherwise), as to render it unfit for the continued vitality of the organism. The disinfectants, mercuric salts and phenols, used in this investigation form chemical compounds with proteins. It would not therefore be surprising that disinfection should conform to a law known to govern many chemical processes, and we may employ one of the well known interpretations as to why these reactions should be gradual and not sudden, viz., that at a particular time only a proportion of the molecules (of the bacteria in the case of disinfection) are temporarily in such a state as to permit of the combination[1]. With *B. paratyphosus* we must presuppose the existence of another factor in addition. In this case it would appear that certain individuals are permanently more sensitive to the reaction than others, or, in other words, possess less resistance to the disinfecting process. The less resistant organisms would then be killed in greater proportion than the more resistant during the earlier stages of the reaction, and the value of K would progressively diminish The nearer one could approach to uniformity of resistance, the more nearly constant would the value of K become (this being very nearly realised in the case of an emulsion of anthrax spores, especially after they have been subjected to a temperature of 80° C.).

In the case of a 24 hours' culture of *B. paratyphosus* permanently different resistances to disinfectants may conceivably be bound up with variation in age. In any collection of bacteria of differing resistance the velocity of disinfection will be proportional to the number of low resistant individuals present. This number, if age is the condition determining resistance, will again be a function of the total number present[2], and the course of the reaction will be expressed thus:—

$$-\frac{dn}{dt} = Kn \times f(n),$$

---

[1] This theory has received substantial support in the case of some radioactive substances, whose decay proceeds in accordance with the unimolecular law (see Rutherford, *Radio-activity*, pp. 182 and 338, 4th ed., 1904).

[2] The decrease in the value of K in the case of *B. paratyphosus* is a regular and orderly one. If values of K are plotted against numbers of surviving individuals, a continuous curve is obtained, showing that the value of K is altering in accordance with some law and bears some relation to the number of surviving bacteria.

instead of

$$-\frac{dn}{dt}=Kn\left(\text{or, on integration, } K=\frac{1}{t_2-t_1}\log\frac{n_1}{n_2}\right),$$

which is the equation referring to the case where the individuals are of uniform resistance.

These hypotheses were confirmed by experiment.

If $f(n)=n^0$, the first equation becomes equal to the second one, and for this, as has been seen, the value of K in every case progressively decreased.

If $f(n)=n^1$, the equation becomes $-\frac{dn}{dt}=Kn^2$, and on integration we get $K=\frac{1}{t_2-t_1}\left(\frac{1}{n_2}-\frac{1}{n_1}\right)$; applying this equation, the value of K was found in every case to progressively increase (see Tables III, VIII and XI).

Between the two values $f(n)=n^0$ and $f(n)=n$ a large series was tried, where $f(n)$ was placed equal to n raised to a series of powers between 0 and 1, *e.g.*, $n^{0\cdot2}$, $n^{0\cdot4}$, $n^{0\cdot5}$, $n^{0\cdot7}$, etc. In many cases the value of K was found to remain fairly constant when $f(n)$ was made equal to $n^{0\cdot5}$ (*e.g.*, Tables III, IV and XI). The differential equation then has the form

$$-\frac{dn}{dt}=Kn^{1\cdot5},$$

and on integrating

$$K=\frac{1}{0\cdot5}\cdot\frac{1}{t_2-t_1}\left(\frac{1}{(n_2)^{0\cdot5}}-\frac{1}{(n_1)^{0\cdot5}}\right).$$

In some cases (*e.g.*, Table VIII), a constant value for K was obtained when $f(n)=n^{0\cdot7}$, *i.e.*

$$-\frac{dn}{dt}=Kn^{1\cdot7},$$

and

$$K=\frac{1}{0\cdot7}\cdot\frac{1}{t_2-t_1}\left(\frac{1}{(n_2)^{0\cdot7}}-\frac{1}{(n_1)^{0\cdot7}}\right).$$

In some cases (Tables V and VII) the value of $f(n)$ lay between n^0 and $n^{0\cdot5}$; putting $f(n)=n^{0\cdot5}$ one obtained an ascending value for K.

The exact value of $f(n)$ was found to depend on details of the materials used, on the exact admixture of the different resistances in any collection of individuals. It depended upon the age of the culture and age of the seed material used for making the culture, and upon the exact period of the disinfection process which was under examination. The value, for example, may be different if K is calculated with a trustworthy control enumeration as n_1, or if the initial number for calculation is the amount surviving after some time has elapsed.

This reasoning was further tested as follows: if the above hypotheses are true, then if by any means a number of *B. paratyphosus* organisms of exactly the same age could be obtained, the truth should again be expressed by the original formula,

$$-\frac{dn}{dt} = Kn,$$

or

$$K = \frac{1}{t_2 - t_1} \cdot \log \frac{n_1}{n_2}.$$

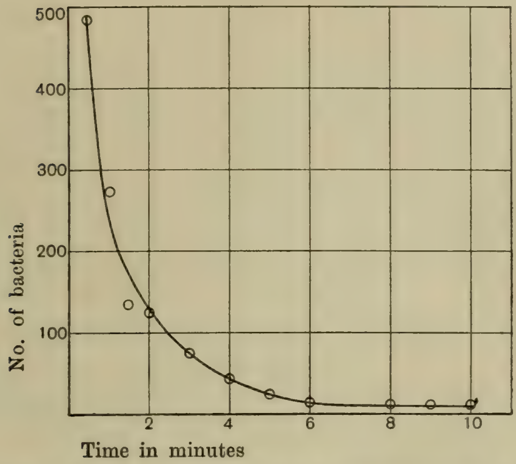


Fig. 9. (Exp. 6. 5. 07.) Disinfection of a 3 hours' culture of *B. paratyphosus* with phenol, 6 per 1000, at 20° C. (see Table XI).

TABLE XI. (*Exp.* 6. 5. 07.)

B. parat. 3 hours' culture. Phenol, 6 per 1000. 20° C.

Time minutes	Mean no. of bacteria present in 1 drop of disinfection mixture	Values of K, assuming reaction to be in accordance with the equations		
		(a) $-\frac{dn}{dt}=Kn$	(b) $-\frac{dn}{dt}=Kn^{1\cdot5}$	(c) $-\frac{dn}{dt}=Kn^2$
0	1000, taken as the initial value of n $(=n_1)$ in calculating values of K			
0·5	484	0·62	0·056	0·0021
1	272	0·56	0·058	0·0026
1·5	136	0·58	0·072	0·0042
2	128	0·46	0·056	0·0034
3	72	0·38	0·058	0·0043
4	46	0·33	0·058	0·0052
5	24	0·32	0·078	0·0081
6	16	0·30	0·072	0·0085
8	13	0·23	0·062	0·0095
9	10·5	0·22	0·062	0·0105
10	11	0·20	0·054	0·0090

This condition cannot be satisfied completely, but approximations can be attempted by using very young cultures. An experiment made with a 3 hours' culture (see Table XI) inoculated in the ordinary way,

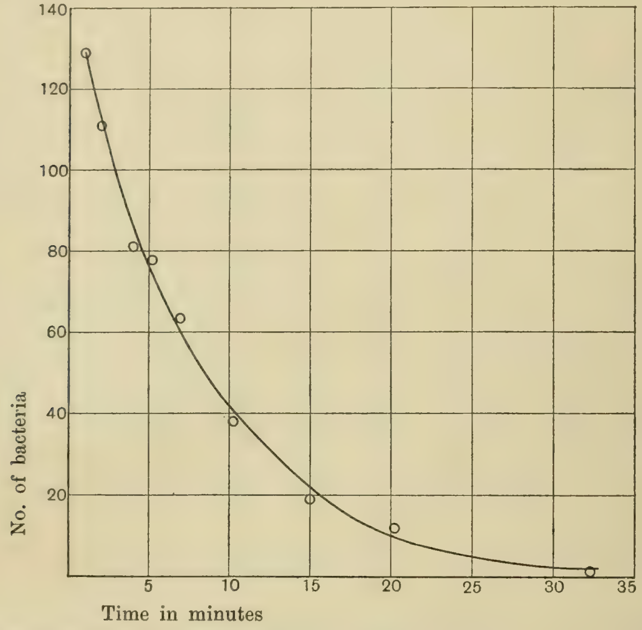


Fig. 10. (Exp. 30. 5. 07.) Disinfection of young individuals of *B. paratyphosus* with phenol, 6 per 1000, at 20° C. (see Table XII).

TABLE XII. (*Exp.* 30. 5. 07.)

B. parat. 3rd generation of young cultures. Phenol, 6 per 1000. 20° C.

Time mins.	Amount of sample taken	Numbers counted on plates	Mean	Mean no. of bacteria present in 1 drop disinfection mixture	K, assuming reaction to be in accordance with the equations	
					(a) $-\frac{dn}{dt}=Kn$	(b) $-\frac{dn}{dt}=Kn^{1\cdot5}$
1	1 drop	147, 142, 99	129	129, taken as initial value of n $(=n_1)$ in calculating values of K		
2	1	114, 106, 112	111	111	0·066	0·014
3	1	94, 99, 97	97	97	0·062	0·013
4	1	100, 91, 57	83	83	0·064	0·015
5·1	2 drops	157, 182, 132	157	78	0·053	0·012
7	3	186, 215, 177	193	64	0·051	0·012
10·1	5	177, 190, 196	188	38	0·058	0·016
15	5	102, 141, 48	97	19	0·059	0·020
20·3	10	145, 154, 55	118	11·8	0·054	0·021
32·3	10	55, 42, 15	37	3·7	0·049	0·028

showed no essential difference from those made with 24 hours' cultures. In the next attempt the relatively large number of veterans added in the seed culture was avoided by passing the organism through several generations in broth, and sub-culturing with small amounts every three hours. It was thus possible to work with young cultures of the third and fourth generation.

The results of experiments with phenol are given in Tables XII—XVI, and it will be seen that the value of K given by the expression

$$K = \frac{1}{t_2 - t_1} \log \frac{n_1}{n_2},$$

although still decreasing in value during the experiments, does not decrease in value nearly so rapidly as was the case with 24 hours' cultures. The alternative equation

$$K = \frac{1}{0{\cdot}5} \cdot \frac{1}{t_2 - t_1} \left(\frac{1}{(n_2)^{0{\cdot}5}} - \frac{1}{(n_1)^{0{\cdot}5}} \right), \text{ integral of } -\frac{dn}{dt} = Kn^{1{\cdot}5},$$

applicable in many of the latter instances gave values of K which increased in value as the experiment proceeded ([illegible] XIV and XV a). The experiment in Table X [illegible] XIII, XIV and XV a, results only are stated.

It is therefore clear that the age of the [illegible] determining its resistance to the action of a disinfectant. In the experiments (Tables XII—XV) made with young individuals, the value of the velocity constant is comparatively small, and approximates to that obtained towards the end of the reaction in more mixed cultures (*e.g.*, compare Table XII, initial value of $K = 0{\cdot}066$, and falling to $0{\cdot}049$, with Table III, initial value of $K = 0{\cdot}27$, falling to $0{\cdot}06$: concentration of phenol in both experiments the same, 6 per 1000: temperature in both instances, 20° C.).

This shows that it is the younger individuals which possess the higher resistance for disinfectants.

TABLE XIII. (*Exp.* 7. 5. 07.)

B. parat. 2nd generation of young cultures. Phenol, 6 per 1000. 20° C.

Time minutes	Mean no. of bacteria present in 1 drop of disinfecting liquid	Values of K, assuming reaction to be in accordance with the equations $(a)\ -\frac{dn}{dt}=Kn$	$(b)\ -\frac{dn}{dt}=Kn^{1{\cdot}5}$
1	1125, taken as initial value of $n\,(=n_1)$ in calculating values of K		
2	418	0·43	0·038
3	212	0·36	0·038
5	47	0·34	0·058
7	20	0·29	0·064

TABLE XIV. (*Exp.* 23. 5. 07.)

B. parat. 3rd generation of young cultures. Phenol, 6 per 1000. 20° C.

Time minutes	Mean no. of bacteria in 1 drop of disinfection mixture	*K*, assuming reaction to be in accordance with the equations	
		(a) $-\frac{dn}{dt}=Kn$	(b) $-\frac{dn}{dt}=Kn^{1\cdot5}$
1	166, taken as the initial value of $n(=n_1)$ in calculating values of *K*		
1·9	125	0·13	0·026
3·0	95	0·13	0·028
5·3	39	0·15	0·038
7·1	33	0·11	0·032
10	16	0·10	0·034
28	1·2	0·08	0·06

TABLE XV. (*Exp.* 27. 6. 07.)

B. parat. 4th generation of young cultures. Phenol, 6 per 1000. 20° C.

	Time minutes	Mean no. of bacteria present in 1 drop of disinfection mixture	*K*, assuming reaction to be in accordance with the equations	
			(a) $-\frac{dn}{dt}=Kn$	(b) $-\frac{dn}{dt}=Kn^{1\cdot5}$
(*a*) 20° C.	1	135, taken as initial value of $n\,(=n_1)$ in calculating values of *K*		
	3	93	0·081	0·018
	5	61	0·086	0·020
	7·5	33·6	0·093	0·026
	10·2	28·3	0·072	0·022
	12·3	25·2	0·065	0·020
	15	16·6	0·065	0·022
	20	10·3	0·059	0·024
	25·3	11·6	0·044	0·017
	30	8	0·042	0·022
	45	0·5	0·055	0·060

TABLE XV. (*Continued.*)

B. parat. 4th generation of young cultures. Phenol, 6 per 1000. 30° C.

	Time minutes	Mean no. of bacteria present in 1 drop of disinfection mixture	*K*, assuming reaction to be in accordance with the equation $-\frac{dn}{dt}=Kn$
(*b*) 30° C.	1	341, taken as the initial value of $n\,(=n_1)$ in calculating values of *K*	
	2	166·5	0·31
	3	76	0·33
	5·4	8·4	0·38

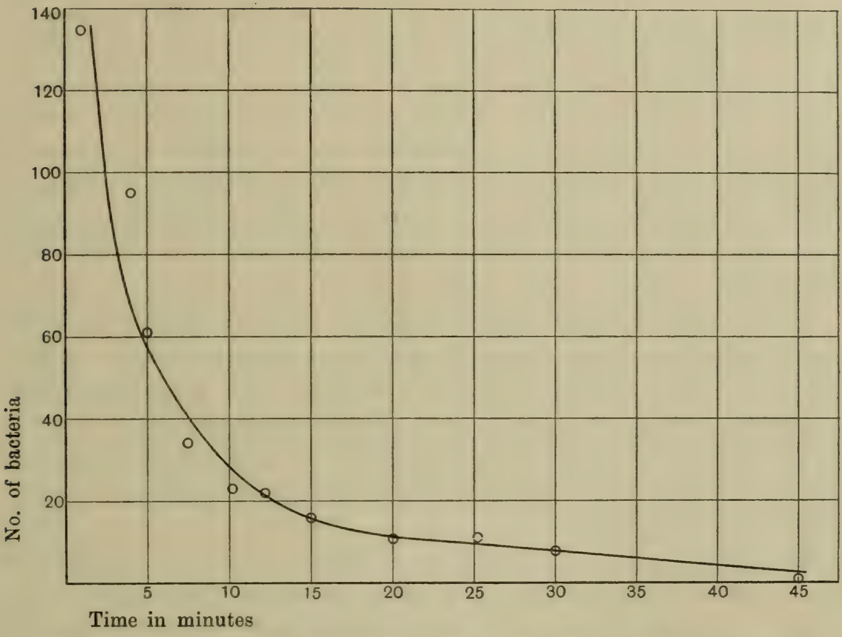


Fig. 11. (Exp. 27. 6. 07.) Disinfection of young individuals of *B. paratyphosus* with phenol, 6 per 1000, at 20° C. (see Table XV *a*).

An ideal case of disinfection, such as that of anthrax spores, may therefore be supposed, with experimental support, to proceed in accordance with the equation $-\frac{dn}{dt} = Kn$, an equation exactly similar in form to that expressing the course of a unimolecular reaction, or reaction of the first order. This latter equation is deduced directly from the Law of Guldberg and Waage: that the velocity of any reaction is at any moment proportional to the active mass of reacting substance present at that moment. For the case of disinfection one may then formulate a similar law: that the velocity of disinfection at any instant is proportional to the number (or weight) of living bacteria present.

An interesting parallel with the case of disinfection is found in such a reaction as that of the inversion of cane sugar with a dilute acid. This reaction is obviously dimolecular, so that the velocity, at any instant, should be proportional to the product of the active mass of the two reagents concerned, sugar and water. Inversion of cane sugar is, however, ordinarily so arranged that one reacting substance, the water, is present in so great an excess that one may consider its concentration to remain constant during the course of the reaction, and the reaction velocity at any moment to be determined only by the active mass of the second reagent, the sugar, and hence to follow the law deduced for a unimolecular reaction. In a similar way in these experiments with disinfection the one reagent, the disinfectant, is present in an excess extremely great in comparison with the weight of bacterial substance present. The latter therefore governs the velocity of the process which in its nature shows an interesting analogy with that of a unimolecular reaction.

In the case of a non-sporing organism such as *B. paratyphosus*, experiments show that the reaction cannot be represented by this simple law, but that a permanent difference in resistance exists amongst individuals of different ages. The younger individuals possess greater resistance. When the conditions are so modified as to operate with individuals of approximately the same age the divergence largely disappears.

The gradual course of the disinfection process has been considered[1] to be completely explained by permanent variations of resistance in the bacteria employed, but it seems more probable that these differences in resistance are less significant than this, but sufficient to modify a process which would otherwise follow the above definite and constant logarithmic law.

Summary of Section I.

1. Disinfection is a process showing close analogy with a chemical reaction, the disinfectant representing one reagent, and the protoplasm of the bacterium the second.

2. It is a gradual process, without any sudden effects, and if the disinfectant is sufficiently dilute to admit of a reasonable time being taken for the process, the reaction velocity can be studied by enumerating the surviving bacteria at successive intervals of time.

3. In the case of disinfection of anthrax spores the reaction proceeds according to the well known equation for a unimolecular reaction embodying Guldberg and Waage's law. In this case "numbers of

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[1] G. Bellei (1904), in the course of experiments on disinfection, repeatedly found that the greater the number of bacteria (*Staph. pyog. aureus*) disinfected, the longer was the time taken by the process. He explained the phenomena as due entirely to differences in resistance. One would rather explain it by considering it in the light of a reaction, where if more of the less abundant reagent be employed the time taken to complete the reaction will naturally be greater also.

surviving bacteria" are inserted in place of "concentration of reacting substance," *i.e.*, the reaction velocity at any moment is proportional to the number of organisms surviving at that moment,

$$-\frac{dn}{dt}=Kn. \quad \text{Values of } K\left(=\frac{1}{t_2-t_1}\log\frac{n_1}{n_2}\right),$$

calculated from the results of experiments, remained constant during the whole time of disinfection. This was the case with values calculated from experiments of

(*a*) Krönig and Paul, working with mercuric chloride, and using the "garnet" method.

(*b*) Madsen and Nyman, working with mercuric chloride and heat, and using Krönig and Paul's method.

(*c*) The present work, using phenol as disinfectant, a simple emulsion of spores, and the method described in the present work.

4. The process, although really involving two "reagents," follows the law of a unimolecular reaction, because the second reagent, the disinfectant, is present in so great an excess, comparatively, that its concentration may be regarded as unaltered during the process. An interesting analogy is thus offered with the case of the inversion of sugar, which, in reality a dimolecular process, obeys the laws relating to a unimolecular reaction for a similar reason.

5. Experiments with cultures of *B. paratyphosus* show a departure from this simple law, the reaction velocity diminishing during disinfection more rapidly than is accounted for by the fall in number of the surviving bacteria. This was the case with each of the three types of disinfectant used.

This divergence is due to differences in resistance between individuals of the various ages contained in such cultures.

SECTION II. THE EFFECT OF VARYING THE CONCENTRATION OF A DISINFECTANT UPON ITS GERMICIDAL ACTION.

Perhaps the best method to employ in investigating the effect of varying concentrations of disinfectant would be the comparison of the velocity coefficients of a series of different concentrations, using exactly similar groups of bacteria. This method is, however, almost impossible, owing to the labour and length of time that would be occupied by any one set of experiments. Another difficulty is that the reaction velocity, in the case of vegetative forms of bacteria, is changing in value throughout the course of the disinfection, so that the velocity coefficients for a particular part only of the process, would be comparable. Accordingly, the method was adopted of measuring the time taken for almost complete disinfection of a constant number of bacteria, this time being considered to be inversely proportional to the mean reaction velocity of the process. This method justified itself by the concordant results which were obtained and has the practical advantage of yielding information specially with regard to the more resistant organisms of the culture. Exactly comparable experiments with a series of different strengths of disinfectant were made. The observations were made simultaneously and the same culture was used.

The method of experiment, except for a few details, was very similar to that of Rideal and Walker. One important difference was the introduction, as unit of measurement, of the drop from the standard pipettes (see p. 96) and the employment of much larger samples in the test cultures (4 drops = 0·08 c.c.). The experiments, moreover, extended over all lengths of time, whereas those of Rideal and Walker were confined to concentrations giving disinfection within fifteen minutes.

The same three types of disinfectants were again investigated and the organism generally used was *B. paratyphosus*, though a few results were also obtained with *Staphylococcus pyogenes aureus.*

A culture of standard resistance was obtained as follows: stock cultures upon sloped agar were maintained at room temperature, and from these a standard loopful was inoculated into 6 c.c. of standard broth and incubated exactly 24 hours at 37° C. This culture was immediately cooled to about 10° C. and then used for experiment.

5 c.c. of disinfectant solution was always employed and to this was added from a standardised pipette 5 drops (0·085 c.c.) of the broth culture, or about 30,000,000 organisms. A series of tubes were prepared containing different concentrations of disinfectant. These were inoculated as nearly as possible at the same time and maintained in a thermostat at constant temperature, viz., 20° C.

At suitable intervals of time samples, 4 drops from the standard pipettes (0·08 c.c.), were withdrawn, added to tubes of dulcite or glucose broth and at once incubated at 37° C. If the sample had contained any living organisms, growth (as evidenced by acid and gas production in either medium in the case of *B. paratyphosus*, and acid production in glucose broth in case of *Staph. pyogenes aureus*) was usually apparent in 24 hours; the tubes were, however, always kept for four days.

The sample taken (·08 c.c.) represented about 1/60 of the total liquid present in the disinfection tube; a negative result in the test culture indicated therefore that about 30,000,000 bacteria had been reduced to less than 60 individuals. The times measured refer therefore to this practical end-point, which represents almost complete disinfection.

TABLE XVI.

Phenol. *B. paratyphosus.* 20° C. 5 drops (·085 c.c.) of a 24 hours' broth culture (about 30,000,000 bacteria) added to 5 c.c. disinfectant solution.

	Parts phenol per 1000 $=C$	Time taken for disinfection, *i.e.* reduction in number of about 30,000,000 individuals to less than 60 $=t$	Values of expression $\frac{1}{C_0 - C_n}\log\frac{C_n t_n}{C_0 t_0}$*, where initial concentration of phenol, $C_0=12$, initial time, $t_0=3{\cdot}25$
Exp. 21. 6. 06	12	3·25 minutes	—
	11	5·5	0·19
	10	8·5	0·17
	9	20·5	0·22
	8	68	0·28
	7	126	0·27
	6	240	0·26
	4·5	?19 hours	—
	4	?19	—
	3	75	0·28
Exp. 6. 6. 06	8	45 minutes	$C_0=8$; $t_0=45$
	7·5	75	0·39
	7	105	0·31
	6·5	125	0·24
	6	225	0·29
	5·5	440	0·29
	5	11·5 hours	0·33

* These and all subsequent values of the expression are calculated with Briggs' logarithms.

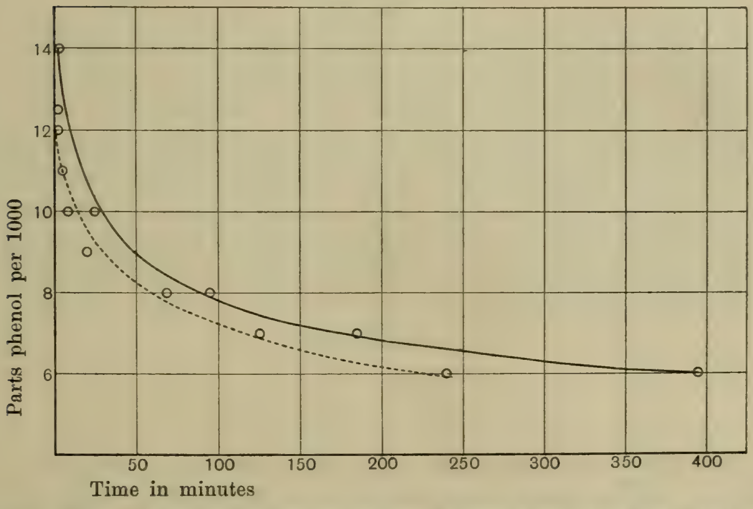

Fig. 12. Effect of varying the concentration of phenol upon the time taken for disinfection of (*a*) *B. paratyphosus*, dotted curve (Exp. 21. 6. 06, Table XVI): (*b*) *Staph. aureus*, continuous curve (Table XVII).

(1) *Phenol.*

The results of experiments with phenol are given in Table XVI (*B. paratyphosus*) and Table XVII (*Staph. pyogenes aureus*) and shown graphically in Fig. 12. The disinfection times given in the tables are the means between the time of last positive and first negative sub-culture. In some cases, where skipping occurred, each end-point was separately determined and the result given is the mean of the separate determinations. In these and similar tables where more than one experiment is given they were always made at different times with other but quite similar cultures.

TABLE XVII.

Phenol. *Staphylococcus pyogenes aureus.* 20° C.

Phenol parts per 1000 $=C$	Time taken for disinfection $=t$	Values of expression $\frac{1}{C_0 - C_n} \log \frac{C_n t_n}{C_0 t_0}$, where initial value of C, $C_0 = 14$, initial value of t, $t_0 = 4{\cdot}5$
14	4·5 minutes	—
12·5	2·5	—
10	25	0·15
8	95	0·16
7	186	0·20
6	395	0·20
4	23·7 hours	0·19

It is evident that within the range observed small variations in concentration of disinfectant caused large differences in the time of disinfection. The relation between these concentrations and the corresponding times when plotted (Fig. 12, ordinates = concentrations of disinfectant: abscissae = times of disinfection) make continuous curves of similar form in the case of either organism. The curves suggested a logarithmic relation and the following equation, arrived at with the kind assistance of Dr J. C. G. Ledingham, was found to apply with very fair accuracy:

$$(\text{a constant})^C = Ct,$$

or

$$\frac{1}{C_0 - C_n} \log \frac{C_n t_n}{C_0 t_0} = \text{a constant},$$

where t_0 and t_n are times for disinfection corresponding to concentrations C_0 and C_n. This equation was obtained in an empirical manner; its marked symmetry, however, suggests that it also has some physical significance.

The values of this expression calculated from the experiments were approximately constant (see Tables XVI and XVII). Considering the difficulty in accurately determining end-points in a process so gradual towards the end as disinfection was shown to be in Section I, the agreement with the above formula is maintained too well to be explained by mere coincidence.

TABLE XVIII.

Disinfectant "A." *B. paratyphosus.* 20° C.

	Parts "A" per 10,000 $=C$		Time taken for disinfection $=t$	Values of expression $\frac{1}{C_0-C_n}\log\frac{C_nt_n}{C_0t_0}$, where initial value of C, $C_0=10$ and initial value of t, $t_0=1{\cdot}5$
Exp. 13. 9. 06	10		1·5 minutes	—
	8		2·5	—
	7		9	0·21
	6		12·5	0·17
	5		162	—
	2·5		more than 4 days	—
	1		,, ,, ,,	—
Exp. 18. 9. 06	5	simultaneous experiments	44 minutes	—
	5		210	—
	5		75	—
	5		105	—
Exp. 19. 9. 06	5·5		110 minutes	—
	5		205	—
	4·5		365	—
	4		500	—
	3		more than 5 days	—

TABLE XIX.

Disinfectant "A," another sample. *B. paratyphosus.* 20° C.

Parts "A" per 10,000 $=C$	Time taken for disinfection $=t$	Values of expression $\frac{1}{C_0-C_n}\log\frac{C_nt_n}{C_0t_0}$, where initial value of C, $C_0=10$, initial value of t, $t_0=1{\cdot}25$
10	1·25 minutes	—
8	1·75	—
7	5	0·15
6	30	0·26
5	84	—

(2) *Disinfectant "A."*

In the case of disinfectant "A" (see Tables XVIII and XIX) there was only a small range of concentrations over which the corresponding times of disinfection could be conveniently measured, *e.g.*, a concentration of 10 in 10,000 disinfected in 1·5 minutes, while 2·5 in 10,000 disinfected in 4 days.

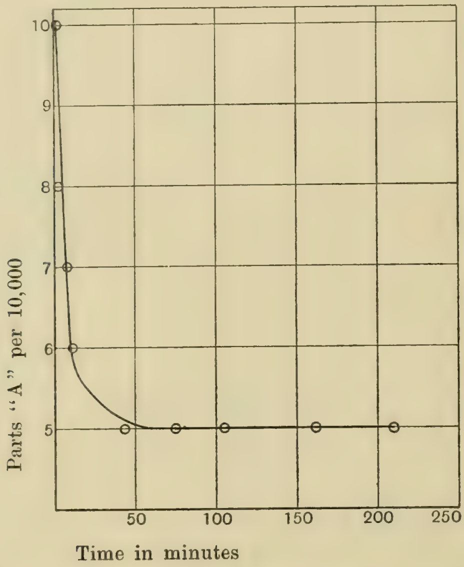

Fig. 13. (Exp. 13. 9. 06 and 18. 9. 06.) Effect of varying concentration of disinfectant "A" upon the time taken for disinfection of *B. paratyphosus* (Table XVIII).

The relation between concentration and time taken is different from that obtaining in the case of phenol. With disinfectant "A" (see Fig. 13) dilution has comparatively little effect upon the course of disinfection up to a certain point (*e.g.*, "A" 10 per 10,000 to "A" 5 per 10,000), then suddenly the curve becomes horizontal and the end-points of the reaction are in consequence difficult to determine accurately (see Table XVIII, Exp. 18. 9. 06). Two different samples of "A" were examined and both showed the same properties.

The number of determinations that can be made with any accuracy are necessarily limited, but if the portion of the curve between the two asymptotes be examined the values of the expression

$$\frac{1}{C_0 - C_n} \log \frac{C_n t_n}{C_0 t_0}$$

remains fairly constant in value (Table XVIII, Exp. 13. 9. 06, and Table XIX).

(3) *Metallic Salts* (mercuric chloride $HgCl_2$, and silver nitrate $AgNO_3$).

Salts of the heavy metals, even when present in a nutrient medium in extremely small amount, exert an inhibitory influence upon the growth of bacteria. This fact was observed by Koch (1881), Behring (1890), Geppert (1889 and 1891) and others. The extent of this inhibition may be gathered from the fact that in my own experiments with mercuric chloride a concentration of 1 in 1,000,000 in the culture medium was found to prevent growth when about 20 individuals (*B. paratyphosus*) were added; a concentration of silver nitrate of 2 in 1,000,000 prevented growth when about 40 individuals were added.

In the case of phenol and the disinfectant "A" this phenomenon of inhibition by low concentrations of the disinfectant was found to be negligible. Control experiments, which were undertaken to test this point, showed that in the case of the most concentrated solutions employed, no error was introduced by the carrying-over of the disinfectant in making the test culture.

In the case of mercuric chloride, or silver nitrate, however, it is essential that the metal be thrown out of solution at the moment of sampling, and, with all but the most dilute solutions, it was necessary to add a large excess of precipitant over and above the amount actually required for the reaction. This fact, noticed by Geppert (1889 and 1891) is well shown in Table XX, where the time taken for disinfection by a definite concentration of mercuric chloride is seen to vary in proportion to the excess of precipitant employed.

TABLE XX.

Effect of adding varying excess of ammonium sulphide to neutralise mercuric chloride on sampling.

Concentration of mercuric chloride in disinfection tube	Amount of AmHS necessary to neutralise $HgCl_2$ carried over	Excess AmHS added over and above * necessary amount	Apparent time taken for disinfection
1 in 1000	0·4 drop	1·00 drop	less than 7 minutes
1 in 1000	0·4	2·00 drops	25 minutes
1 in 10,000	0·04	0·96 drop	less than 5 minutes
1 in 10,000	0·04	1·96	29 minutes
1 in 10,000	0·04	2·96 drops	56 ,,
1 in 10,000	0·04	3·96	56 ,,

* An even number of drops was actually added in each case, the AmHS solution being suitably diluted when necessary.

In addition to the inhibitory action upon the growth of bacteria exercised by traces of metallic salts, there is another phenomenon exhibited by this class of disinfectant. If bacteria are subjected to the action of 1 in 1000, 1 in 10,000, or even weaker solutions of mercuric chloride, there is an interval during which some at least of them may be resuscitated by the timely administration of an antidote (in this case a sulphide solution), but if this antidotal treatment is not employed, no amount of dilution beyond the limits where inhibition occurs can prevent the death of the organism. It would seem that the mercuric salt has been already absorbed by the bacterium and possibly formed some combination with its substance, not, however, to a sufficient extent to prevent recovery if a large excess of the sulphide solution be employed.

The following two experiments illustrate this phenomenon :

Experiment I. 20. 3. 07. (*a*) A 24 hours' broth culture of *B. paratyphosus* was so diluted that in one standard drop there were 20 organisms. One such drop was added to tubes containing 10 c.c. glucose broth together with varying small amounts of mercuric chloride ; inhibition took place when concentration of mercuric chloride in the culture tube reached 1 in 1,000,000.

(*b*) 5 c.c. of 1 in 1000 mercuric chloride solution was placed in the thermostat at 20° C. and inoculated with about 16,000,000 organisms (5 drops of broth culture). The complete disinfection tested by means of sub-cultures precipitated with H_2S, was found to take at least 5 minutes. Between 0·5 and 1·25 minutes after the start, samples of 4 drops (·08 c.c.) were added to

(1) 900 c.c. glucose broth,

(2) 900 c.c. glucose broth, containing 0·15 c.c. H_2S solution.

In (2), 24 hours' incubation showed abundant formation of acid and gas ; in (1) no growth was apparent after many days incubation.

Experiment II. (*a*) A drop from a diluted culture, containing 25 bacteria (*B. paratyphosus*), was added to 900 c.c. glucose broth to which 4 drops (·08 c.c.) $HgCl_2$ 1 in 1000 had previously been added. The result on 24 hours' incubation was abundant acid and gas formation. A similar experiment with 50 organisms gave the same result.

(*b*) About 16,000,000 bacteria of the same culture, *B. paratyphosus*, were added to 5 c.c. of 1 in 1000 mercuric chloride at 20° C. The disinfection was found to take 7·5 minutes. About one minute after the start 4 drops (·08 c.c.) were added from the disinfection tube to

(1) 900 c.c. glucose broth,

(2) 900 c.c. glucose broth, containing ·15 c.c. H_2S solution.

The result was just the same as that of Exp. I : (1) remained sterile, while in (2) acid and gas were apparent after 24 hours' incubation.

In these two experiments, the dilution of 0·08 c.c. mercuric chloride (1 in 1000) by 900 c.c. of broth produced a concentration of mercuric chloride in the broth of 1 in 11,000,000, a concentration which did not produce inhibition (Exps. I (*a*) and II (*a*)) even when very few bacteria were present. After a sojourn of one minute in 1 in 1000 mercuric chloride, exactly similar conditions of sub-culture failed to reveal the existence of any living bacteria (Exps. I (*b* 1) and II (*b* 1)), although the sub-cultures received a comparatively large number of organisms, which could have recovered their vitality if sulphide had been administered as an antidote (Exps. I (*b* 2), II (*b* 2)).

A difficulty in using large excess of these sulphide precipitants is that they themselves are, to a certain degree, inhibitive to bacterial growth. The following plan was therefore adopted in every case, whatever the concentration of mercuric chloride. A constant amount of precipitant was used, viz., the amount shown by separate experiments (with very few, about twenty, organisms) to be the maximum that could be safely employed without fear of inhibition.

The two sulphides generally used were yellow ammonium sulphide and hydrogen sulphide water. Solutions of these sulphides are unstable, hence they were always titrated before use (AmHS with $HgCl_2$ solution, lead acetate paper being used as an indicator; H_2S water directly with standard iodine solution). Sodium and potassium sulphides were also tried, but proved unsuitable owing to the solubility of mercury sulphide in the excess necessarily employed. According to the precipitant used, whether yellow ammonium sulphide or hydrogen sulphide, the disinfecting power of mercuric chloride was found to be very different in amount, and it appeared a question whether the power of the precipitant to decompose the bacteria-mercury compound was being determined rather than the disinfecting power of the original mercuric chloride.

Tables XXI and XXII show the effect of varying the concentration of mercuric chloride upon the time taken for disinfection, when the precipitants used are yellow ammonium sulphide and hydrogen sulphide respectively, and it will be seen that the germicidal value of mercuric chloride is different in the two instances. Yellow ammonium sulphide (Table XXI), *in a certain condition,* would appear to be a more effectual antidote for sublimated bacteria than hydrogen sulphide (Table XXII). It is, however, very difficult to obtain ammonium sulphide in precisely the right condition and it is very unstable. Possibly a certain amount of polysulphide should be present so that the small amount of dissolved mercury sulphide may contain no Hg^{++} ions. On account of this uncertainty ammonium sulphide was abandoned and hydrogen sulphide adopted as a standard precipitant. A saturated solution in distilled water (= $\frac{1}{2}$ normal) was easily prepared and kept in small stoppered bottles in the dark. 0·15 c.c. of this solution to 10 c.c. glucose broth was found to be a sufficient excess to precipitate any of the strengths of sublimate worked with and to exercise no inhibition (tested with 10—20 organisms) on its own account.

TABLE XXI.

Mercuric chloride. *B. paratyphosus.* 20° C. Precipitant, yellow ammonium sulphide.

	Parts $HgCl_2$ per 1000	Time taken for disinfection
Exp. 12. 12. 06	50	4·5 minutes
	10	17
	1	27
	0·1	>127
Exp. 14. 12. 06	10	17
	1	56

TABLE XXII.

Mercuric chloride. *B. paratyphosus.* 20° C. Precipitant, H_2S (0·15 c.c. saturated solution added to each sub-culture tube containing 10 c.c. glucose broth).

	Parts $HgCl_2$ per 1000	Time taken for disinfection $= t$	Nos. expressing concentration of Hg^{++} ions (Luther & Kahlenberg)	$\frac{1}{C_0 - C_n} \log \frac{C_n t_n}{C_0 t_0}$, where initial concentration of Hg^{++} ions, $C_0 = 63$, initial time, $t_0 = 1·5$
Exp. 27. 2. 07	1	1·5 mins.	63	—
	0·5	7	57·5	*
	0·1	13	42·5	0·037
	0·05	10	†37	0·023
	0·01	65	†23	0·030
	0·005	230	†16·5	0·034
Exp. 2. 3. 07	1	2·5	63	$C_0 = 63$, initial time, $t_0 = 2·5$
	0·5	7	57·5	*
	0·1	9·5	42·5	0·020
	0·05	22	†37	0·027
	0·03	57	†33	0·036
	0·01	>150	—	—
	0·005	347	†16·5	0·034
	0·001	>450	—	—

* Numbers obtained were not concordant, probably due to errors in measuring short times of disinfection.

† Numbers obtained by extrapolation.

The examination of the results obtained (see Tables XXI and XXII) shows that when the method of the present research is employed, lower values are obtained for the germicidal value of $HgCl_2$ than those of Koch (1881), Behring (1890), and Geppert (1889). From Table

XXI the astonishing fact is discovered that in a 24 hours' culture of *B. paratyphosus* some individuals at least are able to manifest vitality after contact with 5 °/₀ mercuric chloride for 4 minutes, when ammonium sulphide is immediately applied as an antidote. When hydrogen sulphide is used, mercuric chloride is also seen (Table XXII) to have a considerably lower germicidal value than has usually been attributed to it. The difference in the results shown in Tables XXII and XXI is due to the different antidotal powers of hydrogen sulphide and the particular sample of ammonium sulphide employed. This again is doubtless due to different efficiency of the two reagents in decomposing a fairly stable compound formed by mercuric chloride and some con-

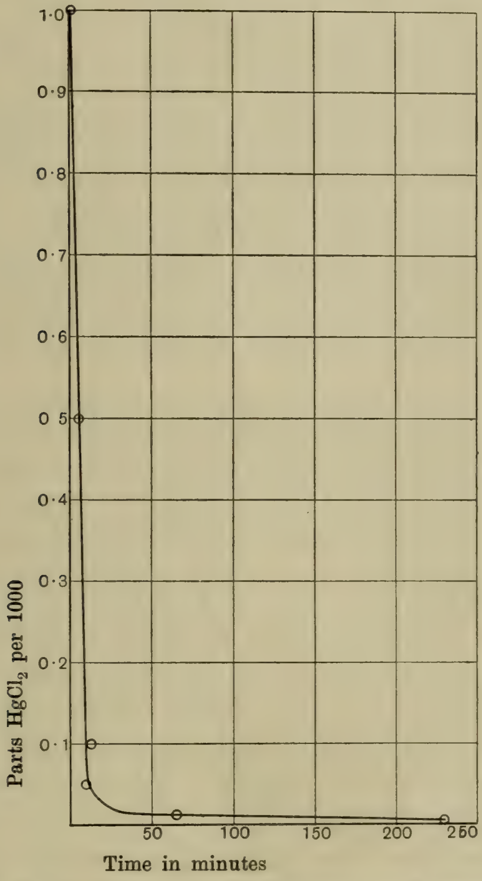

Fig. 14. (Exp. 27. 2. 07.) Times taken for disinfection of *B. paratyphosus* with varying concentrations of mercuric chloride, H_2S being used as precipitant (Table XXII).

stituent of the bacterium. The value of corrosive sublimate as a practical disinfectant, however, remains unassailed.

The effect of altered concentration upon the time taken for disinfection when hydrogen sulphide is the precipitant is graphically shown in Fig. 14. The curve is very different in form to that obtained for phenol, and the formula $\frac{1}{C_0 - C_n} \log \frac{C_n t_n}{C_0 t_0} =$ constant, was found to be quite inapplicable. Confirming an idea originally put forward by Dreser (1893), Krönig and Paul (1897) and Paul and Prall (1907) have published experiments showing that in the case of mercury salts, the mercuric ion[1], rather than the salt, is the real disinfecting agent. These workers employed *Staphylococcus pyogenes aureus* and anthrax spores and showed: (*a*) Mercuric salts, when arranged in order either of their electrolytic dissociation or of their disinfecting value, form two similar series. (*b*) Any procedure which diminishes the dissociation of mercuric chloride, such as the addition of sodium chloride or the substitution of alcohol for water as solvent, also diminishes the disinfecting power.

It therefore seemed probable that the difference in the relation between concentration and reaction velocity in the case of phenol and mercuric chloride might disappear if the mercuric ions were regarded as the real disinfecting agent. I am indebted to Dr N. T. M. Wilsmore of University College for figures representing the concentration of Hg^{++} ions corresponding to various concentrations of mercuric chloride, obtained from the results of Luther (1904) and Kahlenberg (1901). As the determinations of the latter worker were made at 95° C. they were reduced to the required temperature. The combined results of both workers were then plotted and from the curve values were obtained corresponding to the concentrations of mercuric chloride here employed; unfortunately only extrapolation values were available for the lower concentrations. In Table XXII values of the expression $\frac{1}{t_n - t_0} \log \frac{C_n t_n}{C_0 t_0}$ are given, where numbers representing concentrations of mercuric ions are substituted for concentrations of mercuric chloride, and it will be seen that a fair constancy is maintained, showing that disinfection by mercuric chloride is not very unlike that by phenol when the real agent is taken into account.

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[1] That H^+ ions are the real toxic agents in disinfection by the mineral acids appears probable from the experiments of Bial (1897 and 1902) and Winslow and Lockridge (1906).

Silver nitrate. The validity of the arguments given above can also be tested by investigating the relation between concentration and rate of disinfection in the case of such a metallic salt as silver nitrate. Here the electrolytic dissociation in dilute solution is so complete that the concentration of metallic ions will be in proportion to the concentration of the salt.

In working with silver nitrate the procedure had to be modified owing to precipitation of the silver by the trace of broth introduced with the bacteria. An emulsion of bacteria in distilled water was therefore substituted for the drops of broth culture and it was obtained as follows: from the stock culture a broth culture was inoculated with a standard loopful and incubated for 24 hours at 37° C.; from the broth culture a standard loopful was inoculated upon sloped agar tubes and also incubated for 24 hours at 37° C. This agar culture was emulsified with 2 c.c. of distilled water, filtered through muslin and centrifugalised for about 45 minutes. The water was removed, the deposit again emulsified with 2 c.c. distilled water, again filtered and shaken with glass beads. This emulsion when diluted fifteen times with distilled water was found to contain a concentration of bacteria about equal to that of the standard 24 hours' broth culture. Five standard drops were used for each experiment and found to contain 20—40 million bacteria.

The silver salt carried over in the test cultures was found to be most conveniently precipitated as sulphide; 0·15 saturated solution of H_2S in water was added to each tube of glucose broth containing 10 c.c.

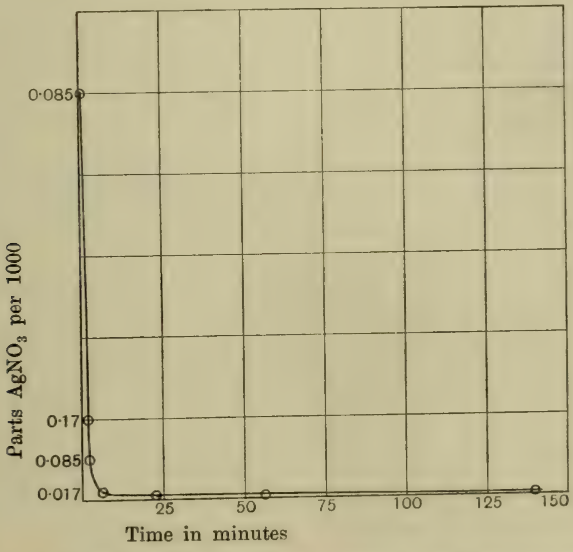

Fig. 15. (Exp. 24. 4. 07.) Times taken for disinfection of *B. paratyphosus* with varying concentrations of $AgNO_3$, H_2S being used as precipitant (Table XXIII).

The results are given in Table XXIII, where also are shown the values of the expression $\frac{1}{C_0 - C_n} \log \frac{C_n t_n}{C_0 t_0}$, and these show a very constant value. This constancy, in a case where the concentration of Ag^+ ions may be taken as approximately proportional to the concentration of the silver salt, is an interesting confirmation of the theory substantiated in the case of mercuric chloride, viz., that in cases of disinfection by metallic salts it is the metallic ion that is the real disinfecting agent.

TABLE XXIII.

Silver nitrate. *B. paratyphosus.* 20° C. About 30,000,000 washed bacteria added to 5 c.c. $AgNO_3$ solution.

	Concentration $AgNO_3$			
	Parts per 1000	Proportional nos. (0·17 per 1000 = 1) = C	Time taken for disinfection = t	$\frac{1}{C_0 - C_n} \log \frac{C_n t_n}{C_0 t_0}$*, where initial concentration, $C_0 = 5$, initial time, $t_0 = 0{\cdot}75$
Exp. 24. 4. 07	0·85	5	0·75 min.	—
	0·17	1	1·5	0·10
	0·085	0·5	2·5	0·11
	0·017	0·1	6·5	0·15
	0·0085	0·05	22·5	0·10
	0·0017	0·01	56	0·16
	0·00085	0·005	140	0·14
	0·00017	0·001	more than 6·5 hrs.	—
Exp. 29. 3. 07	0·85	5	0·75	$C_0 = 5$, $t_0 = 0{\cdot}75$
	0·017	0·1	6·5	0·15
	0·0085	0·05	21	0·11
	0·0017	0·01	69	0·15
	0·00085	0·005	122	0·16

* The values of the expression $\frac{1}{C_0 - C_n} \log \frac{C_n t_n}{C_0 t_0}$ are here all negative in sign, the time taken for disinfection increasing so slowly with decreasing concentration of $AgNO_3$.

The above logarithmic formula was also found applicable to the case of disinfection of anthrax spores with mercuric chloride on further examination of the figures of Krönig and Paul (1897), already referred to. In their recent paper Madsen and Nyman (1907) give a table (p. 390), in which values of K, the velocity constant, are deduced for a series of Krönig and Paul's experiments. The velocity constant of any reaction being inversely proportional to the time taken for the completion of the whole or a definite fraction of the reaction, the above expression $\frac{1}{C_0 - C_n} \log \frac{C_n t_n}{C_0 t_0}$ may be also written $\frac{1}{C_0 - C_n} \log \frac{C_n K_0}{C_0 K_n}$.

In Table XXIV are given the values of K (from Madsen and Nyman) corresponding to different concentrations of mercuric chloride. Numbers expressing the corresponding concentrations of Hg^{++} ions were again deduced from the results of Luther and Kahlenberg; fortunately there was no need of extrapolation in this case. In column 4 are given values of the expression $\frac{1}{C_0 - C_n} \log \frac{C_n K_0}{C_0 K_n}$, numbers representing concentration of Hg^{++} ions being inserted for those of $HgCl_2$, and very good constancy is maintained.

TABLE XXIV.

Mercuric chloride. Anthrax spores. 18° C. Krönig and Paul's experiments.

Parts $HgCl_2$ per 1000	Value of velocity constant $K\left(= \frac{1}{t_2 - t_1} \log \frac{n_1}{n_2}\right)$ (Madsen & Nyman)	Nos. expressing concentration of Hg^{++} ions (Luther & Kahlenberg)	$\frac{1}{C_0 - C_n} \log \frac{C_n K_0}{C_0 K_n}$, where initial concentration of Hg^{++} ions, $C_0 = 88{\cdot}5$, initial value of K, $K_0 = 0{\cdot}22$
16·9	0·22	88·5	- -
8·4	0·14	83·0	0·031
4·2	0·08	76·8	0·032
2·1	0·07	69·0	0·023
1·1	0·026	61·0	0·028

As regards the effect of concentration of disinfectant upon disinfection velocity, there is no longer the close analogy shown in Section I to exist between disinfection and a chemical process involving two reagents which conforms to the unimolecular type, owing to the presence of one constituent in large excess. Whereas in the latter instance a simple proportionality exists between reaction velocity and concentration of reagent, the relation between velocity of disinfection and concentration of disinfectant was found to be a logarithmic one. The range of concentrations studied was necessarily limited to the cases where the corresponding times of disinfection could be measured with accuracy; many of these concentrations were approaching the limit of efficient action, where any chemical process involved is possibly reversible. It is conceivable that a simple proportion might still have been found to exist with higher concentrations; such an investigation is experimentally impossible, owing to the very short time that would be occupied by the reaction. Against this view, however, one may cite the experiments of Krönig and Paul, with anthrax spores and more concentrated solutions of mercuric chloride, which are discussed in the preceding paragraph.

Summary of Section II.

1. When phenol is used as a disinfectant a logarithmic relation exists between its concentration and the time taken for disinfection (the expression $\frac{1}{t_n - t_0} \log \frac{C_n t_n}{C_0 t_0}$ remaining constant in value). This was found to be true in the disinfection both of *B. paratyphosus* and of *Staphyl. pyogenes aureus.*

2. The same relation exists in the case of an emulsified disinfectant and *B. paratyphosus*, but only within narrow limits of concentration.

3. With silver nitrate and *B. paratyphosus* the above logarithmic relation holds good. In the case of mercuric chloride it is also true, if, in place of concentration of mercuric chloride, numbers are inserted representing concentration of Hg^{++} ions. This is shown in the case both of anthrax spores and *B. paratyphosus.* These facts lend further confirmation to the theory that, in the case of disinfection by metallic salts, the metallic ion is the real disinfecting agent.

4. The relation, expressed in 1, between velocity of disinfection and concentration of disinfectant, forms a marked contrast to the simple proportionality obtaining in the case of a chemical reaction of the unimolecular type, with which otherwise (see Section I) disinfection shows a close analogy.

5. Very small traces of salts of the heavy metals were found to prove inhibitive to bacterial growth. Bacteria which had been immersed in such solutions were therefore treated with sulphides when making test cultures. Large excess of sulphide was found indispensable, and, in the case of mercuric chloride, this is probably needed for the splitting up of some compound between the metal and the substance of the bacterium, which will prevent all further growth, however great dilution with culture medium be employed. If, however, a large excess of sulphide is administered subsequently as an antidote, the bacterium may recover its vitality.

SECTION III. THE INFLUENCE OF TEMPERATURE UPON THE VELOCITY OF DISINFECTION.

Robert Koch (1881) showed that the disinfection of anthrax spores with carbolic acid vapour was much more quickly completed if the temperature were raised; Henle (1889) showed the same to be true in the case of disinfection of *B. typhosus* with both phenol and creolin, and

Behring (1890) published the results of a few similar experiments made by Hünermann with mercuric chloride. Heider (1892) was the first to make a series of systematic experiments upon the subject, but as the temperatures employed lay chiefly between 40° C. and 80° C., it is probable that a mixed effect was being investigated, and that some at least of the disinfection was due to heat alone. Madsen and Nyman (1907) find that the velocity of disinfection of anthrax spores by mercuric chloride increases with rise of temperature in accordance with the well-known equation of Arrhenius, the increase being about 2·5-fold for a rise in temperature of 10° C. between the temperatures 25° C. and 45° C.

With the exception of a few experiments with anthrax spores, the work about to be described was all done with *B. paratyphosus*. The method generally employed was similar to that used in Section II, viz., a comparison of the times taken for (almost) complete disinfection at the different temperatures in question. In some cases, however, a comparison of the velocity constants of disinfection at various temperatures was made. This involves series of enumeration experiments, which are exceedingly laborious, and by means of which one can compare the reaction velocities corresponding to, at most, two temperatures. Some of the enumeration experiments in Section I were made simultaneously at different temperatures, and can be made use of in this particular.

Anthrax spores. In Section I (Tables I and II), the velocity constant during disinfection of anthrax spores has been shown to remain constant in value throughout the whole reaction, hence the values found at any two temperatures are perfectly comparable. Two experiments were made with 5 % phenol at temperatures 20·2° C. and 33·3° C. respectively, and the velocity constant of disinfection at 20·2° C. (Table I) was ·047, and at 33·3° C. (Table II) 0·44. Assuming that the velocity of disinfection of anthrax spores with phenol increases in a regular manner with rise of temperature, we get a coefficient for a rise in temperature of 10° C. equal to 5·5, a higher figure than that obtained by Madsen and Nyman for disinfection with mercuric chloride. These workers used the "garnet" method of Krönig and Paul (1897), and obtained their coefficient also by comparison of velocity constants.

B. paratyphosus. A direct comparison of velocity constants in the case of *B. paratyphosus* is much complicated by the fact that, as was shown in Section I, the value of these constants decreases progressively during the course of the disinfection. It is possible, on the other hand, to compare the average velocities of disinfection at different temperatures by determining the times taken for the same number of exactly similar bacteria to be reduced in number by exactly the same amount. This was done by plotting the results of enumeration experiments at two different temperatures, and drawing smoothed curves. Similar points on these curves were subsequently read off and compared. It was essential, for justifiable comparison, that the material disinfected should be exactly similar in either case. The individuals in cultures of *B. paratyphosus* were shown in Section I to possess different resistances to the action of disinfectants. It was therefore necessary that the experiments at different temperatures[1] should be made with the same initial number of bacteria from the same culture, *i.e.*, exactly the same combination of the different resistances.

Two experiments in Section I fulfil these conditions (see Tables VI and VII). The same results are tabulated again in Table XXV *a* and *b* to show the effect of temperature upon disinfection, the times given being obtained from the smoothed curves in Figs. 6 and 7 respectively. The velocity of disinfection of *B. paratyphosus* with 6 per 1000 phenol

TABLE XXV.

Phenol, 6 per 1000. *B. paratyphosus.* 11° C. and 21° C.

No. of bacteria present in one standard drop of disinfection mixture	Time elapsing at 21° C. minutes	Time elapsing at 11° C. minutes	Relative increase in mean reaction velocity for a rise in temperature of 10° C.
Exp. 24. 7. 07. (*a*).	(See Table VI and Fig. 6.)		
900	0	0	—
100	1·0	2·2	2·2
50	1·6	3·3	2·1
10	2·7	6·2	2·3
			Mean 2·2
Exp. 23. 7. 07. (*b*).	(See Table VII and Fig. 7.)		
*	0	0	—
300	1·2	5	4·2
200	1·75	6·5	3·9
100	3	9·5	3·2
10	7·5	20	2·7
			Mean 3·3

* The initial number of organisms was not determined but was the same for experiments at either temperature.

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[1] With an organism as sensitive as *B. paratyphosus* to temperatures above 45° C., the range over which experiments can be made is very limited. At temperatures above 20° C., even with dilute disinfectants, enumeration experiments have to be made with uncomfortable rapidity.

is shown to be increased in the one experiment 2·2 times, in the other 3·3 times, when the temperature is raised from 11° C. to 21° C.

The majority of the experiments were, however, made with the "end-point" method, described in Section II (p. 118), by means of which a comparatively large number of experiments at different temperatures could be made simultaneously.

A series of water baths was maintained at different convenient temperatures, tubes containing 5 c.c. disinfectant solution were placed in them, and inoculated in the usual way with five standard drops of a 24 hours' culture. The disinfection tubes were sampled from time to time, exactly as described in Section II, by withdrawing four standard drops (0·08 c.c.) and adding them to glucose or dulcite broth; the end-points thus determined indicated a reduction of the total number of bacteria to less than 60.

To ascertain whether this less laborious "end-point" method could be substituted for that of comparing the velocity constants at different temperatures, simultaneous comparative experiments were made with exactly similar groups of bacteria. In the one case a comparison was made between the times elapsing at two temperatures before the total number of bacteria was reduced to less than 60, in the other the average reaction velocities at the same two temperatures were compared.

An "end-point" determination was made with a similar culture and similar number of organisms to those of Exp. *a*, Table XXV; unfortunately it was not made simultaneously. Phenol, 6 per 1000, was used, and at a temperature of 21° C. the total number of bacteria was reduced to less than 60 organisms in 6·75 minutes, being the mean of three concordant determinations; at 11° C. the time was 12·5 minutes, the same result being given by three experiments. The coefficient of increase in disinfection velocity for a rise of 10° C. is therefore 1·9. The figure obtained from the enumeration experiment was 2·2 (Table XXV, Exp. *a*).

To test this point further, experiments (Table XXVI) were made employing the two different methods simultaneously upon the same number of bacteria from the same culture. Some difficulty was experienced with the enumeration experiments at 21° C., and only one or two very rapid enumerations were possible before the number of surviving bacteria became too small to be counted with accuracy. The numbers inserted in Table XXVI, 3rd column, are those actually counted at 21° C. The times taken at 11° C., for a corresponding reduction in numbers, were derived from curves drawn through the points obtained by experiment. Figs. 17 *a* and 18 show the course of the latter part

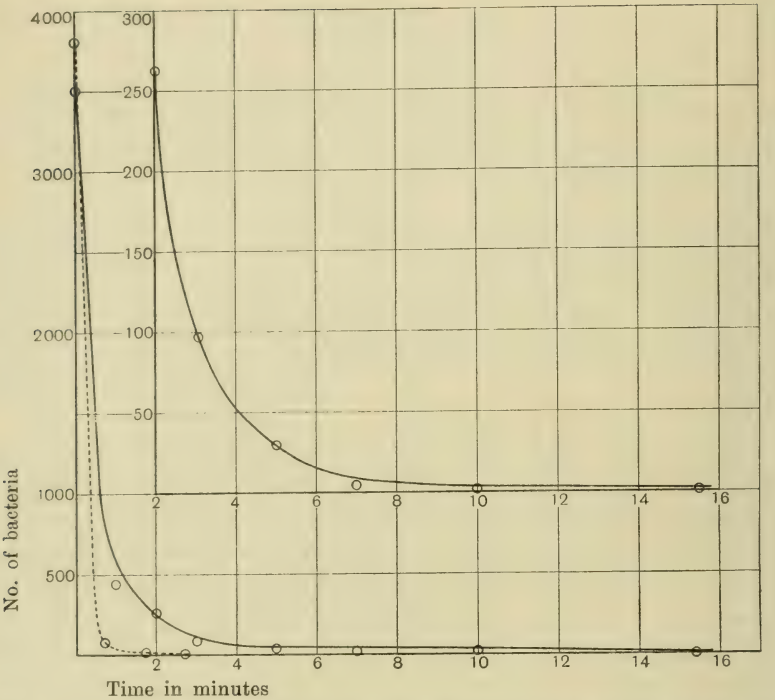

Fig. 17. (Exp. 24. 8. 07.) Disinfection of *B. paratyphosus* with phenol, 8 per 1000, at 11° C., continuous curve: at 21° C., dotted curve.

Fig. 16 *a*. (Exp. 24. 8. 07.) Showing course of the latter part of disinfection of *B. paratyphosus* with phenol, 8 per 1000, at 11° C. (Table XXVI, Exp. (*a*)).

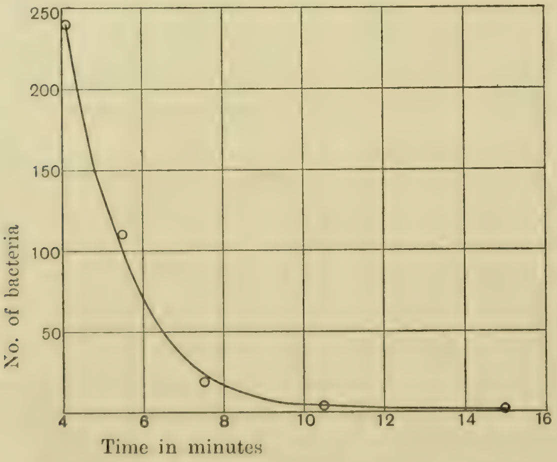

Fig. 18. (Exp. 27. 8. 07.) Showing course of the latter part of disinfection of *B. paratyphosus* with phenol, 8 per 1000, at 11° C. (Table XXVI, Exp. (*b*)).

of the disinfection at 11° C. in the case of Exps. (*a*) and (*b*) respectively, and were used to obtain the times given in Table XXVI, column 4. From this table it will be seen that the numbers obtained by means of the two methods showed close agreement, so that the subsequent employment of the "end-point" method was justified.

TABLE XXVI.

Phenol, 8 per 1000. *B. paratyphosus.* 11° C. and 21° C.

Method of experiment employed	Total no. of bacteria	No. of bacteria present in one standard drop of disinfection tube	Time elapsing at 21° C. minutes	Time elapsing at 11° C. minutes	Relative increase in mean reaction velocity for a rise in temperature of 10° C.
Exp. 24. 8. 07. (*a*).					
	950,000	3,650	0	0	—
"End-point"	60	—	6·5	24·5	3·8
"Enumeration"	—	79	*0·75	†3·35	4·4
Exp. 27. 8. 07. (*b*).					
	3,000,000	13,000	0	0	—
"End-point"	60	—	13·5	41	3·0
"Enumeration"	—	66	*1·75	†6·1	3·5 } 3·05
,,	—	11·4	*3·25	†8·5	2·6

* Times of direct enumeration experiments.

† Times obtained from drawn curves, Figs. 17*a* and 18.

Experiments with B. paratyphosus, illustrating the effect of temperature upon reaction-velocity, in which the "end-point" method was employed.

In these experiments a much larger number of organisms was employed than were used in those in which a direct enumeration was made. The material disinfected was a 24 hours' culture. The increase in the number of organisms produced, when working with phenol, an unexpected rise in the value of the temperature coefficient. This variability according to the number of organisms used was found to be consistent, and the interpretation will be discussed later (p. 148).

1. *Metallic Salts.*

Mercuric chloride. The experiments were carried out exactly as those in Section II, with 20—40 million bacteria from a 24 hours' culture of *B. paratyphosus.* The precipitant used was sulphuretted hydrogen water, 0·15 c.c. of a saturated solution being added to each sub-culture tube containing 10 c.c. glucose broth.

TABLE XXVII.

Mercuric chloride, 1 in 1000. *B. paratyphosus.*

	Temperature degrees centigrade	Time taken for disinfection	Values of A $\left(=\frac{T_0 T_n}{T_0 - T_n} \log \frac{t_2}{t_1}{}^*\right)$, where initial absolute temperature $T_0=293$, and initial time $t_0=2·5$
Exp. 9. 3. 07	42	$<0·5$ min.	—
	29·7	$<0·5$	—
	20	2·5	—
	12·6	4·5	2890
	7·9	6	2590
	0	41·5	4880
			Mean = 3420
			Initial temp. $T_0=293$, ,, time $t_0=1·5$
Exp. 12. 3. 07	26·8	1·5	—
	20	1·5	—
	13·2	3·5	4540
	7·2	11·5	5670
			Mean = 5100

* These, and all subsequent values of this expression, are calculated with Briggs' logarithms.

TABLE XXVIII.

Mercuric chloride, 1 in 10,000. *B. paratyphosus.*

	Temperature degrees centigrade	Time taken for disinfection	Values of A $\left(=\frac{T_0 T_n}{T_0 - T_n} \log \frac{t_n}{t_0}\right)$, where initial absolute temperature $T_0=314·2$, initial time $t_0=1·5$
Exp. 14. 3. 07	41·2	1·5 min.	—
	29·9	2·5	*
	19·8	21	4930
	11·1	65	4850
	6·7	41	3690
	0	124	3990
			Mean = 4360
			$T_0=314·6$, $t_0=0·75$
Exp. 6. 3. 07	41·6	0·75	—
	30·7	2·5	4580
	19·8	11·5	5010
	13·9	36	5480
	6·8	50	4610
	0	101	4390
			Mean = 4810

* Figure is not concordant, probably due to inaccuracy in measuring the short intervals of time.

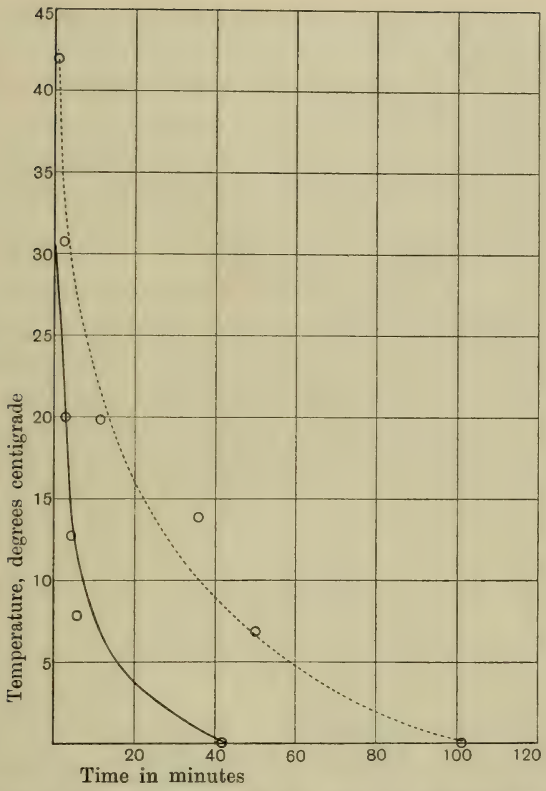

Fig. 19. Influence of temperature upon the time taken for disinfection of *B. paratyphosus* with mercuric chloride. Continuous curve, $HgCl_2$ 1 in 1000 (Table XXVII, Exp. 9. 3. 07): dotted curve, $HgCl_2$ 0·1 in 1000 (Table XXVIII, Exp. 6. 3. 07).

TABLE XXIX.

Mercuric chloride. *B. paratyphosus.*

	Concentration of mercuric chloride parts per 1000	Temperature degrees centigrade	Time taken for disinfection (derived from curve in Fig. 19) minutes	Relative increase in mean reaction velocity for a rise in temperature of 10° C.
Exp. 9. 3. 07, Fig. 19	1·0	25	1·2	
		20	2·7	3·3
		15	4	2·6
		10	7	4·0
		5	16	5·8
		0	41	
				Mean value = 3·9
Exp. 6. 3. 07, Fig. 19	0·1	40	1·2	
		30	4·0	3·3
		20	13·5	3·4
		10	38	2·8
		0	102	2·7
				Mean value = 3·0

The results of two experiments with $HgCl_2$ 1 in 1000 are given in Table XXVII, and of two experiments with $HgCl_2$ 1 in 10,000 in Table XXVIII. Fig. 19 (abscissae = time taken for disinfection: ordinates = temperature of disinfection) shows the results of one set of experiments with either concentration, and the points found by experiment are seen to lie on or near to continuous curves, the velocity of disinfection increasing with rise of temperature in a very orderly manner (see Table XXIX).

Silver nitrate. An emulsion of washed bacteria was used as material to be disinfected in place of the usual drops from the broth culture, and dilutions were so arranged (see p. 129) that each experiment dealt with the disinfection of about 20—40 million bacteria.

The results of experiments with two concentrations of silver nitrate are given in Table XXX, and shown graphically in Fig. 20.

TABLE XXX.

Silver nitrate. *B. paratyphosus.*

	Concentration $AgNO_3$ parts per 1000	Temperature degrees centigrade	Time taken for disinfection	Values of A $\left(=\frac{T_0 T_n}{T_0 - T_n}\log\frac{t_n}{t_0}\right)$, where initial absolute temperature $T_0=313{\cdot}2$ and initial time $t_0=0{\cdot}67$
Exp. 25. 4. 07	0·017	40·2	0·67 min.	—
		30·7	2·6	5900
		20	13·5	5930
		12·25	17·5	4530
				Mean = 5450
				$T_0=314{\cdot}1,\ t_0=4{\cdot}5$
Exp. 21. 6. 07	0·0017	41·1	4·5	—
		33·3	35	*
		20·1	72·5	5280
		16·3	167·5	5740
		1·8	329	4090
				Mean = 5040

* Figure obtained was not concordant.

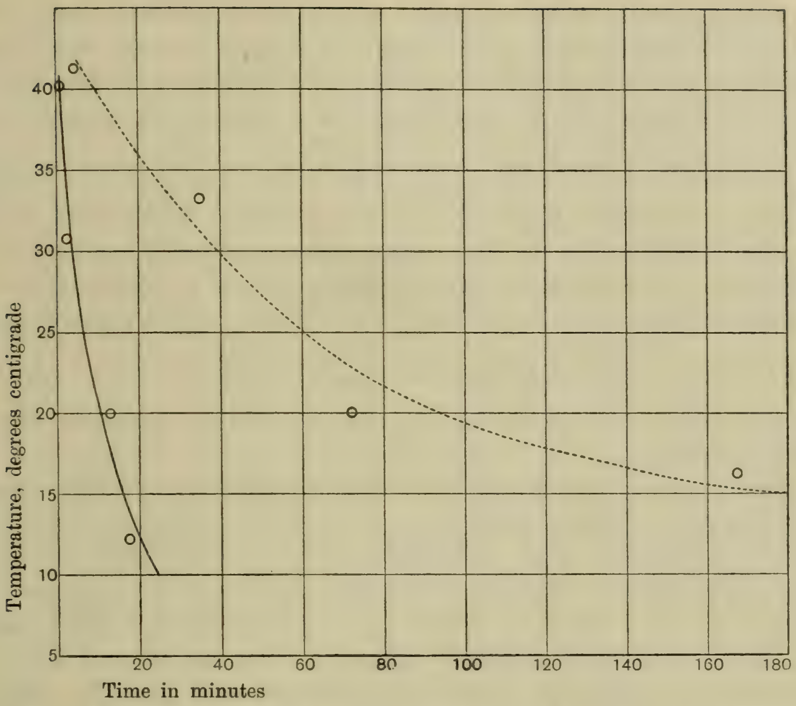

Fig. 20. Influence of temperature upon the time taken for disinfection of *B. paratyphosus* with silver nitrate. Continuous curve, $AgNO_3$ 0·017 per 1000 (Table XXX, Exp. 25. 4. 07): dotted curve, $AgNO_3$ 0·0017 per 1000 (Table XXX, Exp. 21. 6. 07).

TABLE XXXI.

Silver nitrate. *B. paratyphosus.*

	Concentration of $AgNO_3$ parts per 1000	Temperature degrees centigrade	Time taken for disinfection (derived from curves in Fig. 20) minutes	Relative increase in mean reaction velocity for a rise in temperature of 10° C.
Exp. 25. 4. 07, Fig. 20	·017	40	1·2	3·3
		30	4	2·9
		20	10·7	2·3
		10	24·5	
				Mean value = 2·8
Exp. 26. 1. 07, Fig. 20	·0017	40	9·7	
		35	23·5	3·9
		30	39	2·6
		25	60·5	2·4
		20	94·5	3·0
		15	180	
				Mean value = 3·0

The apparently consistent effect of rise of temperature upon the velocity of disinfection points again to a close analogy with the ordinary chemical reaction. The formula of Arrhenius, derived from the more complicated expression of Van t' Hoff, has been found applicable to many chemical reactions, viz., $A = \frac{T_0 T_n}{T_0 - T_n} . \log \frac{K_0}{K_n}$, where A is a constant, and K_0 and K_n are the velocity constants of the reaction in question corresponding to the absolute temperatures T_0 and T_n respectively. Since the time taken for completion of a reaction may be considered as inversely proportional to the velocity of the reaction, one may rewrite the equation thus:—$A = \frac{T_0 T_n}{T_0 - T_n} \log \frac{t_n}{t_0}$, where t_n and t_0 are the times taken to complete[1] the reaction at absolute temperatures T_n and T_0 respectively.

This formula, found by Madsen and Nyman (1907) to apply to the disinfection of anthrax spores with mercuric chloride, is also applicable to disinfection of *B. paratyphosus* with both mercuric chloride and silver nitrate. Values of A, calculated from the results of experiment, are inserted in Tables XXVII, XXVIII and XXX, and are found to be approximately constant. A certain amount of discrepancy does exist, but difficulties doubtless arise from the small amount of inhibition, which cannot be avoided when working with metallic salts.

The increase of reaction velocity with rise of temperature is conveniently expressed as the relative increase per 10° C. rise in temperature. From Figs. 19 and 20 (which like all the subsequent curves in this section were constructed by drawing smoothed curves to include as nearly as possible all the points determined by experiment), numbers were obtained giving a series of values for this temperature coefficient in the case of disinfection by mercuric chloride and silver nitrate. These figures are given in Tables XXIX and XXXI respectively. Values of 2·8 and 3·0 were obtained for two concentrations of silver nitrate, and 3·9 and 3·0 were the values obtained in the case of two different concentrations of mercuric chloride, figures of the same order as those obtained for ordinary chemical reactions.

2. *Phenol.*

In Tables XXXII, XXXIII, XXXIV and XXXV is shown the

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[1] In theory the reaction is completed only after an infinite time. In this instance the reaction is considered at an end, when less than 60 individual bacteria remain undisinfected, *i.e.*, about $\frac{1}{50000}$ of the total number.

TABLE XXXII.

Phenol, 12·5 per 1000. *B. paratyphosus.*

	Temperature degrees centigrade	Time taken for disinfection	Value of $A\left(=\frac{T_0T_n}{T_0-T_n}\log\frac{t_n}{t_0}\right)$, where initial abs. temp. $T_0=294$ and initial time $t_0=2{\cdot}5$
Exp. 25. 10. 06	21	2·5 minutes	—
	14·6	8	*
	2·6	216	8530
	0	460	8640
	– 3	more than 8·25 hours	—
			Mean = 8580

* Figure obtained was not concordant.

TABLE XXXIII.

Phenol, 10 per 1000. *B. paratyphosus.*

	Temperature degrees centigrade	Time taken for disinfection	Value of $A\left(=\frac{T_0T_n}{T_0-T_n}\log\frac{t_n}{t_0}\right)$, where initial abs. temp. $T_0=303{\cdot}5$ and initial time $t_0=1{\cdot}5$
Exp. 24. 9. 06	30·5	1·5 minute	—
	27·5	3·5 minutes	*
	21·3	9	8090
	16·5	27	7870
Exp. 26. 9. 06	15·8	23·5	7130
	6	226	7530
Exp. 25. 10. 06	0	17·5 hours	7910
			Mean = 7710

* Figure obtained was not concordant.

TABLE XXXIV.

Phenol, 8 per 1000. *B. paratyphosus.*

	Temperature degrees centigrade	Time taken for disinfection	Value of $A\left(=\frac{T_0T_n}{T_0-T_n}\log\frac{t_n}{t_0}\right)$, where initial abs. temp. $T_0=306{\cdot}6$ and initial time $t_0=3{\cdot}5$
Exp. 25. 9. 06	42·7	< 1 minute	—
	33·6	3·5 minutes	—
	29	9	8250
	21	47·5	8100
	16·3	94	7330
			Mean = 7890
Exp. 27. 9. 06	21	52·5	—
	16	101	—
	3	more than 23 hours	—

influence of temperature upon disinfection of *B. paratyphosus* in the case of four different concentrations of phenol. The same results are shown graphically in Fig. 21, where the four curves corresponding to the four different concentrations are all seen to be similar in form. The curves have a different slope from those expressing effect of temperature upon disinfection with metallic salts, the influence of temperature being here very much greater. The equation of Arrhenius was, however, applicable to this instance also; values of the expression $\frac{T_0 T_n}{T_0 - T_n} \log \frac{t_n}{t_0}$ are given in Tables XXXII—XXXV, and remain very constant. The

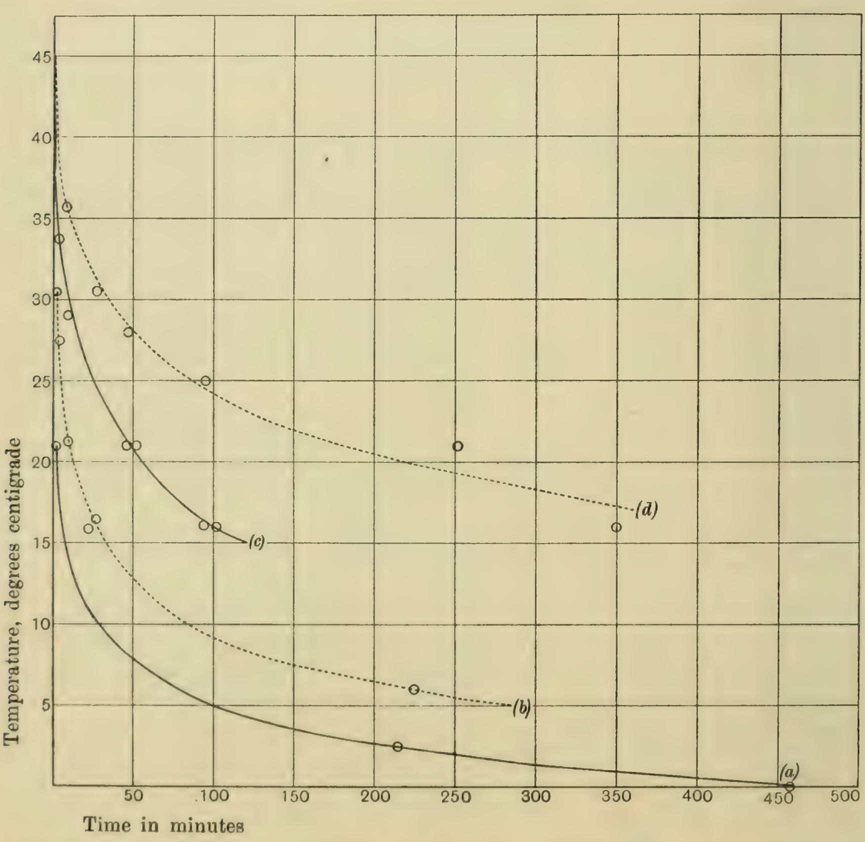

Fig. 21. Influence of temperature upon the time taken for disinfection of *B. paratyphosus* with phenol. Curve (*a*), phenol 12·5 per 1000 (Table XXXII, Exp. 25. 10. 06): curve (*b*), phenol 10 per 1000 (Table XXXIII, Exp. 24. 9. 06 and 26. 9. 06): curve (*c*), phenol 8 per 1000 (Table XXXIV, Exp. 25. 9. 06 and 27. 9. 06): curve (*d*), phenol 6 per 1000 (Table XXXV, Exp. 27. 9. 06 and 1. 10. 06).

TABLE XXXV.

Phenol, 6 per 1000. *B. paratyphosus.*

	Temperature degrees centigrade	Time taken for disinfection	Value of $A \left(= \frac{T_0 T_n}{T_0 - T_n} \log \frac{t_n}{t_0} \right)$, where initial abs. temp. $T_0 = 308 \cdot 7$ and initial time $t_0 = 9$
Exp. 27.9.07	44	< 1 minute	—
	35·7	9 minutes	—
	30·5	27·5	8650
Exp. 1.10.06	28	47·5	8660
	25	95	8750
	21	251	8890
	16	350	7180
	6	more than 10 hours	—
			Mean = 8430
		Phenol, 3 per 1000.	
Exp. 1.10.06	43	131 minutes	—
		Phenol, 1·2 per 1000.	
	44	more than 24 hours	—

TABLE XXXVI.

Phenol. *B. paratyphosus.*

	Concentration of phenol parts per 1000	Temperature degrees centigrade	Time taken for disinfection (derived from curves in Fig. 21) minutes	Relative increase in reaction velocity for a rise in temperature of 10° C.
Fig. 21, curve *a*	12·5	20	2	
		15	7	14·0
		10	28	14·3
		5	100	16·4
		0	460	
				Mean value = 14·9
Fig. 21, curve *b*	10	30	2	
		25	4	6·0
		20	12	8·1
		15	32·5	7·1
		10	85	8·8
		5	285	
				Mean value = 7·5
Fig. 21, curve *c*	8	35	3	
		30	9	8·3
		25	25	6·2
		20	56	4·8
		15	120	
				Mean value = 6·2
Fig. 21, curve *d*	6	35	11	
		30	34	7·9
		25	87	6·5
		20	220	
				Mean value = 7·2

discrepancies occurring in some cases at the higher temperatures are doubtless due to the impossibility of measuring with accuracy the corresponding short intervals of time.

The temperature coefficients of the reaction velocity for an exact difference of 10° C. are given in Table XXXVI, where the times given in the 3rd column were obtained from the curves in Fig. 21. The value obtained for the highest phenol concentration appears to be abnormally high. In the case of the other concentrations the temperature coefficients are more nearly alike and the mean value, 7, is about twice as large as that obtained for disinfection with metallic salts.

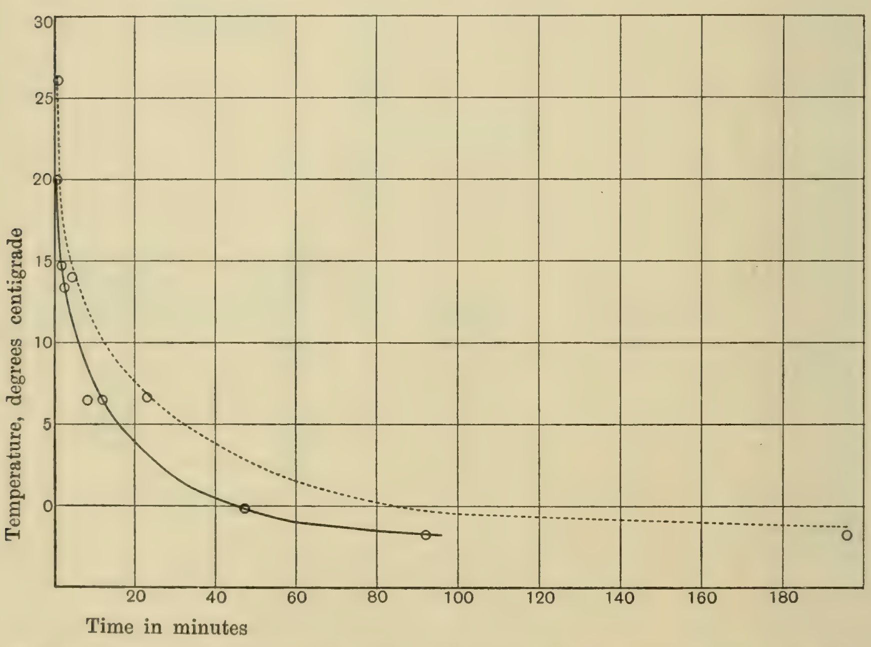

Fig. 22. Influence of temperature upon the disinfection of *B. paratyphosus* with disinfectant "A." Continuous curve, "A" 10 per 10,000 (Table XXXVII, Exp. 24. 1. 07 and 26. 1. 07): dotted curve, "A" 8 per 10,000 (Table XXXVIII, Exp. 2. 2. 07).

3. *Disinfectant "A."*

With regard to the effect of temperature, the disinfectant "A" falls into the same category as phenol. The results of five experiments with two different concentrations of "A" are given in Tables XXXVII and XXXVIII. The formula of Arrhenius was found applicable in this instance also, the expression $\frac{T_0 T_n}{T_0 - T_n} \log \frac{t_n}{t_0}$ remaining constant in value.

TABLE XXXVII.

Disinfectant "A," 10 per 10,000. *B. paratyphosus.*

	Temperature degrees centigrade	Time taken for disinfection minutes	Value of $A\left(=\frac{T_0T_n}{T_0-T_n}\log\frac{t_n}{t_0}\right)$, where initial abs. temp. $T_0=293$ and initial time $t_0=0·75$
Exp. 24. 1. 07	20	0·75	—
	13·4	2·6	6870
	6·7	12	7250
	−0·3	47·5	7090
			Mean = 7070
			$T_0=293,\ t_0=0·87$
Exp. 19. 1. 07	20	0·87	—
	7·9	15	9010
	−1·3	85	7440
			Mean = 8220
			$T_0=293,\ t_0=1·25$
Exp. 26. 1. 07	20	1·25	—
	14·7	1·5	—
	6·6	8·5	7830
	−1·7	92	8710
			Mean = 8270

TABLE XXXVIII.

Disinfectant "A," 8 per 10,000. *B. paratyphosus.*

	Temperature degrees centigrade	Time taken for disinfection minutes	Value of $A\left(=\frac{T_0T_n}{T_0-T_n}\log\frac{t_n}{t_0}\right)$, where initial abs. temp. $T_0=292·9$ and initial time $t_0=6$
Exp. 29. 1. 07	19·9	6	—
	14	18·5	6970
	6	140	7860
	−0·1	200	6080
			Mean = 6970
			$T_0=293·3,\ t_0=0·75$
Exp. 2. 2. 07	26·1	0·75	—
	20·3	0·75	—
	14	4·5	10400
	6·6	23·25	8920
	−1·7	>171	—
			Mean = 9660

The mean reaction velocity (see Fig. 22 and Table XXXIX) was found to increase 7 to 8-fold for a rise in temperature of 10° C., a figure approximating in value to that found for phenol, and again about twice as great as the number obtained in the case of disinfection by metallic salts.

Disinfection by phenol and the disinfectant "A" being thus equally influenced by temperature is evidence of the similarity of the actual process in the case of the two disinfectants.

TABLE XXXIX.

Disinfectant "A." *B. paratyphosus.*

Concentration of "A" parts per 10,000	Temperature degrees centigrade	Time taken for disinfection (derived from curves in Fig. 22) minutes	Relative increase in mean reaction velocity for a rise in temperature of 10° C.
10	20	0·8	
	15	1·5	6·9
	10	5·5	10·7
	5	16	8·2
	0	45	
			Mean value = 8·6
8	25	0·7	
	20	1·5	6·1
	15	4·3	8·3
	10	12·5	7·6
	5	32·5	6·8
	0	85	
			Mean value = 7·1

This high temperature coefficient in the case of phenol was surprising in view of the results obtained from the enumeration experiments given in detail at the beginning of the present section (Tables XXV and XXVI). In those experiments, the mean velocity of disinfection was found to increase two or three, or, at most, four times with a rise in temperature of 10° C. To explain the discrepancy by a criticism of the "end-point" method was not possible, because, as shown above, perfectly concordant results were obtained from parallel experiments using the two methods, the same lower coefficient being obtained in either case (Table XXVI). The only difference between the earlier enumeration experiments and those just described is in the number of bacteria disinfected. In the former case, thousands only of bacteria were taken, in the latter case 20—40 million bacteria were disinfected in each experiment. Simultaneous parallel experiments were therefore made with the "end-point" method, using exactly similar organisms from the same culture, but varying the total number disinfected, and, to make the comparison of results easier, water baths were maintained at temperatures differing exactly 10° C. from one another. The results are given in Table XL, each separate experiment showing the result of two sets of determinations, made over the same range of temperature, and concerned with the disinfection of exactly similar material, the quantity only of bacteria being different in the two cases. It was found to be invariably true that where more bacteria were disinfected, the temperature coefficient was higher (Exps. I, II, III and IV, Table XL).

TABLE XL.

Phenol. *B. paratyphosus.*

	Concentration phenol parts per 1000	No. of organisms added (approximately)	Temp. degrees centigrade	Time of disinfection (reduction of nos. in Col. II to less than 60) minutes	Relative increase in mean reaction velocity for a rise in temperature of 10° C.
Exp. I, 28. 8. 07	10	(*a*) 1,000	21	0·75	3·3
		,,	11	2·5	
		(*b*) 750,000	21	2·5	6·0
		,,	11	15	
Exp. II, 2. 8. 07	8	(*a*) 6,600	21	2·75	8·2
		,,	11	22·5	
		(*b*) 1,030,000	21	25	10·3
		,,	11	257	
Exp. III, 31. 8. 07	8	(*a*) 440,000	21	4·5	6
		,,	11	27·5	
		(*b*) 76,000,000	31	4·5	7·7
		,,	21	34·5	
		,,	11	>283	>8·2
Exp. IV, 2. 8. 07	6	(*a*) 6,600	31	3·5	4·3
		,,	21	15	
		(*b*) 750,000	31	20	10·4
		,,	21	208	

In Section I, it was shown that among the individuals of a 24 hours' culture of *B. paratyphosus*, there existed permanent differences in resistance to disinfection, and that the younger organisms possessed the greater resistance. It therefore seemed possible that, among other differences, there might also be a different temperature coefficient for disinfection of bacteria of different resistances. The greater the total number of bacteria the greater also will be the number of more resistant forms. In any "end-point" method the properties of the more resistant individuals alone are investigated; if then the disinfection of the latter should have a higher temperature coefficient than that of the less resistant individuals, disinfection experiments made with a large number of bacteria would show a higher temperature coefficient than those made with comparatively few[1]. For in this latter instance the presence of a few of the more resistant bacteria with higher temperature coefficient will not be made evident by the experimental method.

TABLE XLI.

Phenol. *B. paratyphosus.*

Concentration of phenol parts per 1000	Nature of culture employed	No. of organisms added (approximately)	Temperature of disinfection degrees centigrade	Time of disinfection (reduction of nos. in Col. III to less than 60) minutes	Relative increase in mean reaction velocity for a rise in temperature of 10° C.
Exp. 6. 9. 07.					
8	24 hrs.' culture	187,000	21	2·25	5·5
	,,	,,	11	12·5	
	,,	56,000,000	21	32·75	12·2
	,,	,,	11	401	
	Young culture	81,500	21	0·8	13·7
	3rd generation	,,	11	11	
Exp. 3. 9. 07.					
6	24 hrs.' culture	110,000	31	3·2	5·5
	,,	,,	21	17·5	3·9
	,,	,,	11	67·5	
					Mean = 4·7
	,,	16,000,000	41	1·75	7·8
	,,	,,	31	13·75	10·3
	,,	,,	21	14·1	
					Mean = 9·05
	Young culture	8,850	31	1·5	8·1
	3rd generation	,,	21	12·25	6·8
	,,	,,	11	83	
					Mean = 7·45

Special experiments were made to investigate the effect of temperature upon the disinfection of young organisms which were shown to possess the higher temperature coefficient. The necessary cultures were obtained by repeatedly sub-culturing in broth after intervals of about 3 hours. For purposes of comparison, simultaneous experiments were made with 24 hours' cultures, themselves taken from the same stock culture as the series of young growths (see Table XLI). Two concentrations of phenol were employed, and, in both cases, it was found that, whereas in the case of bacteria from a 24 hours' culture the high temperature coefficient was given only when a large number was present, an equally high coefficient is shown when only a few younger individuals were being disinfected. This result, combined with the theoretical considerations in the foregoing paragraph, yields additional evidence that, in a culture of *B. paratyphosus* the younger organisms offer the greater resistance to disinfection.

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[1] It seems probable that in the case of disinfection with metallic salts, no such difference exists; a comparatively low temperature coefficient was obtained when large numbers of bacteria were disinfected. Whether this coefficient would be reduced in this case also, when small numbers were disinfected, could only be ascertained by further experiment.

The fact that the younger and more resistant individuals in a culture of *B. paratyphosus* become relatively less resistant if the temperature is raised, is of great practical importance, and suggests an additional advantage in using hot or even warm solutions when disinfecting. In the case of spore-bearing organisms, such as *B. anthracis*, there probably exists no such complication, for the velocity coefficient of disinfection at constant temperature is the same for all individuals and no such difference in resistance can be detected. The temperature coefficients will doubtless be the same also for all the organisms.

TABLE XLII.

Results of Ballner's experiments with saturated steam and anthrax spores.

Temperature degrees centigrade	Time taken for complete sterilisation minutes	Values of $A\left(=\frac{T_0 T_n}{T_0 - T_n}\log\frac{t_n}{t_0}\right)$, where initial abs. temp. $T_0 = 378{\cdot}3$ and initial time $t_0 = 0{\cdot}43$
105·3	0·43	—
104·5	0·66	—
103·3	0·75	16480
102·3	0·9	16110
101·2	1·14	14610
100·7	1·7	18390
99·7	2·5	19190
98·4	2·8	16570
97·45	3·16	15460
96·4	3·3	13890
95·2	4·5	14070
94·2	5	13200
93·3	9	15260
92·7	8·7	14330
91·2	14	14780
90·4	14·7	14150

Some interesting parallel experiments upon disinfection by heat should be mentioned here. Ballner (1902) measured the time taken to kill an approximately constant number of anthrax spores, using saturated steam at different temperatures, and obtained a valuable series of figures (Table XLII). The object of his investigation was a purely practical one, concerning laboratory sterilisation at high altitudes. The formula of Arrhenius was, however, applied to his figures, and will be found to fit them very well, when the error inseparable from the measurement of very short intervals of time is taken into account (see values of "A," Table XLII). Ballner's results are expressed graphically in Fig. 23, where a smooth curve is drawn to include as many experimental points as possible. The velocity of disinfection is seen to be increased about tenfold (see Table XLIII) for a rise in temperature of 10° C., a very high temperature coefficient.

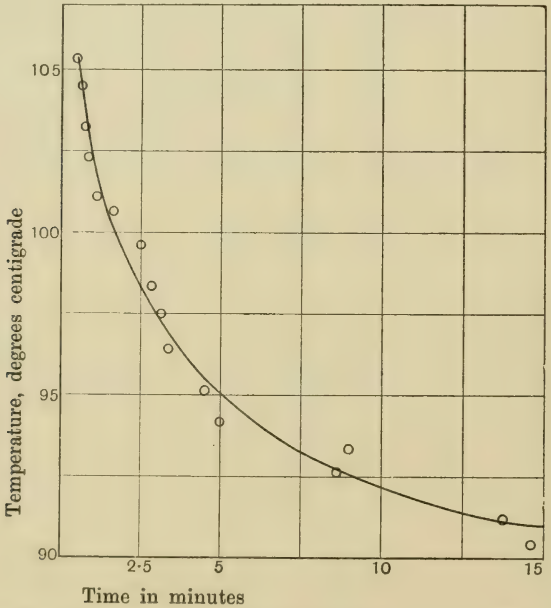

Fig. 23. Sterilisation of anthrax spores with saturated steam at different temperatures, from Ballner's results (see Table XLII).

Similar experiments by Meyer (1906) gave a much lower figure for the disinfection of the spores of *B. subtilis* and *B. robur*, the velocity of disinfection increasing only 4—5 times for 10° C. rise in temperature. Meyer examined his results for theoretical significance and found a logarithmic ratio to exist between the temperature of the experiment and the time taken to kill. The series of temperatures formed terms of an arithmetical progression, while the corresponding times of disinfection (reciprocals of mean velocities of disinfection) were in geometrical progression. Meyer's relation may be expressed thus:

$$\frac{1}{t_1 - t_2} \log \frac{v_1}{v_2} = \text{constant},$$

where v_1 and v_2 are the reaction velocities of disinfection at temperatures t_1 and t_2 respectively.

The formula of Arrhenius has reference to the absolute temperatures involved, thus:

$$\frac{T_1 T_2}{T_1 - T_2} \log \frac{v_1}{v_2} = \text{constant},$$

where v_1 and v_2 are the reaction velocities of disinfection at absolute temperatures T_1 and T_2 respectively.

TABLE XLIII.

Results of Ballner's experiments derived from curve drawn in Figure 23.

Temperature degrees centigrade	Time of disinfection minutes	Relative increase in the mean velocity of disinfection for a rise in temperature of 10° C.
105	0·55	—
104	0·70	—
103	0·80	—
102	1·05	—
101	1·30	—
100	1·65	—
99	2·15	—
98	2·65	—
97	3·27	—
96	4·05	—
95	5·10	9·3
94	6·40	9·2
93	8·10	10·1
92	10·50	10·0
91	14·75	11·3
		Mean = 10·0

The simpler relation is included in the expression of Arrhenius and applies perfectly to the results of Ballner, and also to all those obtained in the present investigation with chemical disinfectants. The values of both constants were calculated in the case of most experiments and equally concordant results were obtained. The question as to which formula should be applied could only be settled by means of experiments over a much larger range of temperature, and this is impossible from the nature of the organism employed. For a range of temperature of only about 40° C., the value of the product T_1T_2 in Arrhenius' expression remains throughout very much the same in value, and the two formulae become almost identical. It might be instructive to make similar investigations with thermophilic bacteria.

Effect of Temperature upon Inhibition.

Behring (1890) showed that the influence of temperature upon inhibition was the exact opposite of that upon disinfection. He found that the growth of anthrax bacilli at room temperature was inhibited when 1 in 400,000 mercuric chloride was present; at 37° C. a concentration of 1 in 100,000 was necessary to inhibit growth. A similar effect was shown by Brooks (1906) in the case of fungi and various poisons.

These somewhat anomalous facts were confirmed by some experiments made during the present work with dilute phenol solutions and *B. paratyphosus*. The concentration of phenol had been reduced to 4 per 1000, in order to obtain a slower rate of disinfection, so that the effect of temperature might be more easily studied with enumeration experiments. The temperatures chosen were 20° C. and 30° C., and the very unexpected result was obtained that disinfection progressed more rapidly at the lower than at the higher temperature, the average value of the velocity constant being about 1·5—3·0 times as great at 20° C., as at 30° C. (Table XLIV).

TABLE XLIV.

Phenol, 4 per 1000. *B. paratyphosus.*

Temperature of experiment	Time elapsing minutes	Mean no. of bacteria present in one standard drop of disinfection mixture	K, assuming reaction to be in accordance with equation $-\frac{dn}{dt}=Kn$
Exp. 16. 7. 07 20° C.	1	685, taken as initial value of n	
	5	143	0·17
	11	175	0·16
	20	3	0·14
30° C.	1	661, taken as initial value of n	
	3	437	0·09
	5	238	0·11
	11	69	0·10
	22	0·66	0·14
Exp. 18. 7. 07 20° C.	1	459, taken as initial value of n	
	2	143	0·51
	3	27·3	0·61
	4	4·7	0·66
	5	1·7	0·56
30° C.	1	519, taken as initial value of n	
	2	464	0·05
	3	274	0·14
	4·4	124	0·18
	5·2	75	0·20
	7	28·5	0·21
	10	6·1	0·20
	15	0·96	0·19

In Section II (Table XVI), it is seen that a concentration of 4 per 1000 phenol took about 20 hours to complete disinfection when 6 per 1000 required only 4 hours. Phenol in the lower concentration may be said to be altering in character from that of a disinfectant to that of a solution able only to produce inhibition.

When bacteria are exposed to the action of weak antiseptics, it is evidently a very great point in their favour if the temperature should happen to be at or near that of their own growth optimum. In the case of strong solutions of disinfectants, the times involved are altogether too short for any effort after growth on the part of the bacteria to be apparent. But in the case of weak disinfectant solutions the organisms are able to make a struggle and are much assisted if the temperature is in the neighbourhood of their own growth optimum. Probably the results shown in Table XLIV would be reversed if the temperatures chosen were say 40° C. and 50° C.; unfortunately such experiments would be useless because at 50° C. temperature itself becomes harmful to such an organism as *B. paratyphosus.*

Summary of Section III.

1. The reaction velocity of disinfection increases with rise of temperature in a manner similar to that of a chemical reaction. The formula of Arrhenius is applicable also to the case of disinfection, the expression $\frac{T_1 T_2}{T_1 - T_2} \log \frac{t_2}{t_1}$ (where t_1 and t_2 represent times taken for disinfection, reciprocals of mean reaction velocities, at absolute temperatures T_1 and T_2 respectively), remaining approximately constant in value.

2. The mean reaction velocity of disinfection with metallic salts increases 2—4-fold for a rise in temperature of 10° C. In the case of phenol and the disinfectant "A" the temperature coefficient is much higher, usually between 7 and 8. These experiments were made with about 20—40 million bacteria from a 24 hours' culture of *B. paratyphosus.*

3. The value of the temperature coefficient for disinfection of *B. paratyphosus* with phenol was found to vary with the number of individuals disinfected. The younger, and more resistant, individuals possess a higher temperature coefficient than the less resistant forms. The value of the coefficient varied from 2 to 10, according to the nature of the bacteria used for the experiment.

4. Inhibition is also influenced by temperature, but apparently in a different manner, being lessened or increased according as the particular temperature is near to or remote from that of optimum growth for the organism used.

GENERAL SUMMARY.

1. A very complete analogy exists between a chemical reaction and the process of disinfection, one reagent being represented by the disinfectant, and the second by the protoplasm of the bacterium.

2. Three classes of disinfectants were studied, (*a*) metallic salts ($HgCl_2$ and $AgNO_3$), (*b*) phenol, and (*c*) emulsified disinfectants (disinfectant "A"). *B. paratyphosus* and spores of *B. anthracis* were chosen as types of vegetative and spore-bearing organisms respectively.

3. In the case of anthrax spores, the disinfection process proceeds in obedience to the well-known equation for a unimolecular reaction, if numbers expressing "concentration of reacting substance" are replaced by "numbers of surviving bacteria."

4. Experiments with *B. paratyphosus* show a departure from the simple law owing to permanent differences in resistance to disinfectants among the individual organisms. The younger bacteria were proved to be the more resistant.

5. The process of disinfection is influenced by temperature in an orderly manner, and the well-known equation of Arrhenius can be applied.

(*a*) Disinfection of *B. paratyphosus* by metallic salts is influenced by temperature to about the same degree as most chemical reactions, the reaction velocity being increased about three-fold for a rise in temperature of 10° C.

(*b*) For disinfection of *B. paratyphosus* by phenol and the disinfectant "A" there was a much higher temperature coefficient, viz., seven to eight. In the case of phenol the effect of temperature was again found to be complicated by the want of uniformity among the individual bacteria. Disinfection of the younger, more resistant bacteria, was found to possess a higher temperature coefficient than that of the less resistant forms, the coefficient varying from ten to three, or two according to the age and number of the bacteria disinfected.

6. It follows from (5) that there is a very great advantage in the use of warm solutions for practical disinfection.

7. Experiments, made with varying concentrations of disinfectant, and using similar groups of bacteria from cultures of *B. paratyphosus*, showed a definite logarithmic relation, between the concentration of disinfectant and the mean reaction velocity of disinfection, to exist in the case of phenol and the disinfectant "A."

8. In the case of silver nitrate, the same relation existed, but, in the case of mercuric chloride, numbers representing concentration of the salt had to be replaced by those representing concentration of the metallic ion. This confirms the theory that in disinfection with metallic salts the metallic ion is the real disinfecting agent.

9. This logarithmic relation is surprising in view of the simple proportionality existing in the case of chemical processes running the course of a unimolecular reaction, with which disinfection shows a close analogy.

10. Some evidence was obtained that, in disinfection with mercuric chloride, a toxic compound is formed between the metal and the substance of the bacterial cell. This compound prevents all further growth, but vitality can be restored by the administration of a large excess of soluble sulphide as an antidote.

I am glad to have this opportunity of expressing my great indebtedness to Dr C. J. Martin, at whose suggestion the work was undertaken, and who has helped me throughout, not only with most valuable advice, but also with practical assistance in many of the experiments.

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ON VARIATION IN WEIGHT OF NORMAL GUINEA-PIGS IN RELATION TO THE ESTIMATION OF FREE DIPHTHERIA TOXIN.

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In the foregoing paper (p. 512) relationships have been established between loss of weight in test guinea-pigs and dose of diphtheria toxin injected. In connection with the above, the normal increase of weight during the period of observation, the effect of the animal ceasing to eat, or any change of diet, had to be taken into consideration. The following note records observations on normal increase of weight, with constant and variable diet, on healthy animals.

Method.

Ninety-four guinea-pigs were sorted out into batches of five individuals (one batch consisting of 4), so that the companion pigs did not differ from one another by more than 5 grams. Each batch was placed in a separate and similar cage and all were fed regularly at 7 A.M. and 4 P.M. daily. The pigs were weighed regularly between 11 A.M. and noon, and the weight of each individual noted daily, a spring balance being used, the divisions on the scale of which would indicate a difference of 5 grams.

Normal Increase in Weight.

All the pigs were fed with oats at 7 A.M. and with cabbage and oats at 4 P.M. for a week. The number of pigs and the mean values for the normal increase are given in Table I for five days, two days being allowed for the animals to become acclimatised to their surroundings, diet, the relatively high temperature prevailing, viz. 16° C. at midnight to about 30° C. at midday, and to recover from a railway journey, diet of unknown character, etc. From the figures it is probable that, for guinea-pigs of 160 to 210 grams initial weight, the rate of increase in weight is approximately the same and is nearly a straight line relation, *i.e.* the increment is proportional to the time. The absolute value of the increment per diem, under the above circumstances, was 7 to 9 grams in the two most divergent series, with the exception of one set, in which only four pigs were classed, where the value is approximately 6 grams. I conclude, therefore, that for guinea-pigs used as indicators of free diphtheria toxin the normal increase in weight will be practically independent of the difference in absolute weight of the test animals, provided this difference does not exceed 60 grams, and further that, as the daily increment probably decreases slowly with increasing absolute weight of the test animals in an hyperbolic relation, the value for guinea-pigs of 250 to 260 gms. will be in the neighbourhood of 6 gms. per diem.

TABLE I.

Average weights of guinea-pigs showing increment during 5 *days.*

No. of pigs	0	1	3	4	5
7	210	216	234	243	245
23	202	210	229	235	241
13	191	200	219	224	228
19	183	190	209	215	219
16	173	180	200	204	209
9	163	170	186	194	203
4	150	151	168	180	180

Effect of Temporary Change of Diet.

Of the entire series of pigs, 29 were retained on the diet above described and 65 pigs were given a single meal in which grass was substituted for cabbage, and then the original food continued for four days. The pigs would not at first eat the grass so that the effect was striking, for the average per pig of the cabbage group increased by 7 gms., 7 gms., 7 gms. and 5 gms. on succeeding days, whereas the grass group decreased by 10 gms. per pig on the first day and on substituting cabbage, increased on the succeeding days by 12 gms., 6 gms. and 6 gms.; being at the end of this period 6 gms. lower in average weight than the pigs of the cabbage group. Unless a constant diet be employed during the test period of diphtheria toxin any relation between lethal dose and weight will be seriously affected.

Effect of Continued Change of Diet.

Many of the pigs originally closely approximating in weight had increased in weight to a much greater extent than others, although subject to the same food conditions, and consequently it was considered advisable to sort out the animals into groups of five, the individuals of each batch differing, as before, by not more than 5 gms. This selection necessitated, for many pigs, a change of cage and of companions, and the effect was that the majority of these pigs lost an average of 5 gms., and in the case of certain animals 10 gms. It will be seen from this that very slight changes in the conditions may interfere with the dose-weight relation. Other slight changes produce equally marked effects. In order to obtain a fairly uniform condition, all the pigs were now kept on cabbage and oats for a week. The cages were then separated into two groups, each containing batches of pigs corresponding closely in weight, the total number of pigs selected for each series being 36. One group was fed on oats and cabbage and the other on oats and grass for seven days. The average weight of each group at the initial weighing being taken as the origin, the total changes in weight for this period are given in Table II.

TABLE II.

Change in weight during 9 days on diet.

A = Cabbage and Oats; B = Grass and Oats.

1	2	3	4	5	6	7	8	9
A 0·5	A 5	A 10·5	A 17	A —	A 21	B 27	B 21	22
B 4	B − 4	B − 1	B 5·5	B —	B 12	A 13·5	A 24	35·5

The figures (Table II) show that the effect of substitution of grass for cabbage is attended by an initial marked diminution in the weight of the animals. After two days the pigs fed on grass begin to increase in weight at the same rate as those fed on cabbage, although owing to the initial depression they show no tendency to attain an equal absolute weight. On the seventh and eighth days the grass group was given cabbage and the cabbage group was placed on a grass diet. The change from cabbage to grass produced a fall on the eighth day, followed by a slow rise on the ninth, whereas the change from grass to cabbage gave rise to a very great increment in weight on the eighth and ninth days, the original grass series being at the end of this period 13·5 gms. heavier than the original cabbage series.

Effect of other Changes in Diet for One Day.

Ten guinea-pigs which had been fed on grass for a week were given dry grass and oats and showed on the following day an increment in weight of 4 gms. per pig.

Ten pigs of similar weights and also accustomed to a grass diet were supplied with dry grass, oats and a liberal supply of water in separate troughs; in this case the average increment was 9·5 gms.

Under the same conditions, dry grass, oats and minced beef gave a rise in weight of only 4 gms.

The pigs did not eat the beef.

Further experiments with beef alone led to a loss in weight of 21 gms., as however it was found that the pigs had not eaten the beef, the result is obviously due to no food ingested.

This decrease of 21 gms. per day is approximately of the same magnitude as the decrements obtained on the injection of lethal doses of solutions containing free diphtheria toxin, which lends support to the view that the latter largely represent starvation curves.

In conclusion, the normal variation of the guinea-pig as regards weight seems, to me, to give an experimental error of at least 10 °/₀ in the estimation of lethal doses from decrement in weight under favourable circumstances, and under ordinary conditions the error is probably two or three times this magnitude.

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ON THE ESTIMATION OF FREE DIPHTHERIA TOXIN:

WITH REFERENCE TO THE RELATIONS EXISTING BETWEEN LETHAL DOSES, LETHAL TIMES AND LOSS IN WEIGHT OF THE GUINEA-PIG.

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THE unit lethal dose of a fluid containing free diphtheria toxin may be defined, by Ehrlich's method of standardisation, as the smallest quantity of the fluid which, diluted to 4 c.c. with saline or water and injected subcutaneously into a healthy guinea-pig of 250 to 280 grams weight, causes death in 4 to 5 days. The standardisation of a toxin is usually made in two ways—(1) various dilutions of the toxin are injected and the unit or minimal lethal dose, M.L.D., obtained; (2) various dilutions of the toxin are mixed with an empirically fixed quantity of antitoxin—the Ehrlich immunisation unit, I.E.[1]—and are injected into a series of guinea-pigs. By this test the amount of toxin necessary to neutralise the unit of antitoxin and still leave a lethal dose in excess, usually denoted as the L+ value of the toxin, is determined. When the quantity of toxin is, in both cases, greater than the unit lethal dose the time required for death or lethal time is shortened, and with smaller quantities of toxin death is delayed beyond the standard time. Further, lethal doses usually produce a daily diminution in the weight of the test animal until death ensues, and sublethal doses give rise to a temporary decrease in weight.

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[1] Immunisierungseinheit.

In a recent investigation of the neutralisation of diphtheria toxin by antitoxin, Arrhenius and Madsen (1904) have deduced a quantitative relation between lethal dose and lethal time, and likewise an empirical connection between dose of toxin and loss of weight in the test animals. If the observations of Arrhenius and Madsen be found to agree with those on other brews of diphtheria toxin obviously standardisations would be rendered more accurate or a saving in animal life could be effected. In their further experiments, designed to test the validity of their views on the toxin-antitoxin reaction, Arrhenius and Madsen find that their observations, interpreted according to the above-mentioned relations, are in close agreement with the theoretically derived values for free diphtheria toxin. Nernst (1904) has pointed out that the equation used by Arrhenius and Madsen is merely an interpolation formula, and the close agreement between calculated and observed results does not prove the validity of their hypothesis. Their observations, however, seem to show that diphtheria toxin is gradually and continuously neutralised by increasing amounts of antitoxin, a result which is in opposition to the experimental investigations and views of Ehrlich (1898), (1903), according to whom the neutralisation takes place in steps owing to the successive combination of several distinct toxic and atoxic substances with different combining affinities present in the toxin. From Arrhenius and Madsen's point of view the step-like neutralisation found by Ehrlich and his pupils is to be interpreted as, in part, due to experimental errors, and the smoothness of the neutralisation curves which they, themselves, have obtained is, in part, due to the more profitable interpretation of their experimental results gained by the application of empirical relations between lethal doses, lethal times, and loss of weight.

It seemed, therefore, to the authors to be important to control the relations deduced by Arrhenius and Madsen, both from the standpoint of toxin and antitoxin standardisation, and the physico-chemical investigation of the toxin-antitoxin reaction by experiments *in vivo*. Fortunately an extensive record of data had been collected by one of us at the Lister Institute of Preventive Medicine during his standardisation of antitoxic sera, within the period 1900 to 1905, and these observations have been subjected to detailed analysis by the other. In this communication the chief features of a preliminary examination of some of these observations are presented, pending an investigation by more refined statistical methods, such as those of Prof. Karl Pearson. One of us, viz. G. Dean, is responsible for the experimental data alone, and the other, J. Craw, for the calculations and conclusions, carried out during his tenure of scholarships from the "Lister Institute," the "British Medical Association" and the "Grocers' Company."

Lethal Doses of Toxin and Time of Death.

In Table I the relations found by Arrhenius and Madsen to hold between lethal doses and lethal times in days, columns 1 and 2, are reproduced. In this connection that quantity of toxin which killed with certainty in 3·3 days was taken as unit toxin or minimum lethal dose, M.L.D., but it is of course easy to calculate proportional series of doses when another time limit is selected for the M.L.D., *e.g.* 3, 4 or 5 days; thus in Table II we give the lethal doses obtained from Arrhenius and Madsen's relation for the second to the sixth day on the basis of a 3-day unit or M.L.D., series "L. dose A. and M." The dotted curve with crosses in Fig. 1 gives a graphical representation of this relation, the lethal doses being shown on ordinates, and the corresponding lethal times on the abscissae. Further, Arrhenius and Madsen assume that if a guinea-pig recovers, its maximum loss of weight will, in general, occur on the fifth day after inoculation and that the loss in weight on any particular day up to the fourteenth will bear a definite ratio to the maximum.

TABLE I.

Toxin-Time Relations. (Arrhenius and Madsen.)

Time	Dose	*R*	Gm.	Dose
1	1·6	40	120	1·30
1·5	1·4	—	100	1·20
2	1·25	52	80	1·10
2·5	1·15	—	65	1·00
3	1·05	70	60	0·92
3·3	1·0	—	55	0·85
3·5	0·97	—	50	0·78
4	0·91	87	45	0·70
4·5	0·85	—	40	0·61
5	0·80	100	35	0·55
6	0·71	98	30	0·50
7	0·64	—	20	0·45
8	0·59	78	10	0·40
9	0·55	—	0	0·35
10	0·51	51	<0	0·35
12	0·45	—	—	—
14	0·40	11	—	—
>14	0·40	—	—	—

Thus, if the maximum loss on the fifth day be taken as 100 the decrement in weight on the first day would have the relative value 40. This series of ratios is reproduced in Table I, column 3 (R), and it is possible, by means of it, to calculate from the loss of weight on the first or the second, third, etc. day, what the maximum loss of weight would be on the fifth day, as the absolute loss on any particular day has merely to be multiplied by a definite known ratio. In this manner the loss in weight on each day, both of pigs which survive and of those which die, gives a calculated value for the maximum for which an average can then be obtained. Arrhenius and Madsen consider that a definite relation exists between the maxima so obtained and the dose of toxin injected, and this is reproduced in Table I, columns 4 and 5. The series of weight ratios is represented diagrammatically in Fig. 2, the abscissae giving the time in days and the ordinates the ratio scale 0 to 100.

TABLE II.

Toxin-Time Relations.

Lethal time in days	2	3	4	5	6
Lethal dose	1·8	1·1	0·8	0·7	0·65
Range of dose	5—1·4	2—1·0	1·2—0·7	0·9—0·5	0·8—0·45
L. dose (A. & M.)	1·2	1·0	0·87	0·76	0·67

The uncertainty associated with the estimation of free diphtheria toxin, owing to variation in the susceptibility of individual guinea-pigs, appears to us to bring these determinations into the class of phenomena which must be treated by more general methods of probability. The deviation from the most probable result decreases as the number of pigs tested increases; thus the problem is to find the likelihood of a result derived from a limited number of tests agreeing approximately with an expected value, provided we proceed on the basis of an hypothesis such as that of Arrhenius and Madsen. If, on the other hand, we proceed according to the method of Ehrlich the probabilities are unknown and we have the inverse problem, viz., to elucidate the M.L.D. and L + values and their theoretical significance from the experimental data. In the present preliminary communication we have adopted the latter method to control the relations between lethal time, dose of toxin and loss of weight. It may, in parenthesis, be remarked that Ehrlich's rectangular method of representation of the neutralisation of toxin by antitoxin would, if carried out systematically, give a better idea of the exact value of the experimental data, but that Arrhenius and Madsen's curves give a better idea of the course of the reaction.

In order to control this relation the data referring to 200 different brews of diphtheria toxin were tabulated, and average values were found for the relation between lethal doses and lethal times. Owing to the large number of estimations the arithmetic and geometric means did not differ appreciably.

The range of dose causing death on any particular day is considerable: for the first day it is very great and no upper limit can be given, for the second to the sixth days the ranges are given in Table II, for durations exceeding seven days no relation whatever could be found. In all cases the values given are the arithmetic means. It may be observed that there are more terms[1] obtainable from any single series of estimations of diphtheria toxin than there are constants, and consequently all the terms must be used in deducing a relation between lethal times and lethal doses. Such a relation has been found for the above-mentioned 200 toxins and is given in Table II, Series 1 and 2, the graphical representation of which is to be seen in the continuous curve of Fig. 1. This graduation curve does not reproduce any of the observations exactly, but it runs evenly through the roughness of the observations and so indicates their general trend. These average values are also probably not a true representation of the experimental data, but only an approximation; it seems to us not improbable that the "mode," *i.e.* the lethal dose which on a particular day most often causes death, is probably a better guide to the toxin-time relation than the "mean," for owing to the spread or scatter of the observations being, in any selected day, unsymmetrical about the mean and congregating during the first, second and thirds days in the neighbourhood of the longer periods, the frequency curves exhibit a pronounced skewness, a matter which will be dealt with in another paper. This may be gathered from Fig. 1, in which the black circles on the continuous curve represent the means, whereas the straight lines corresponding to each circle represent the ranges—the deviation from symmetry is apparently not a negligible factor.

Omitting for the present the toxin-time relation for the first day, it will be seen, from Table II and Fig. 1, that the lethal dose 1·2 obtained by Arrhenius and Madsen for the second day differs considerably from our values, viz. 1·8, but that on the third day the values correspond closely, and on the fourth, fifth and sixth days are practically identical. The relation we have obtained seems capable of simpler expression than that of Arrhenius and Madsen, for we have the approximate relation that

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[1] *i.e.* lethal values for the series of days considered.

Lethal Dose × Lethal Time = Constant.

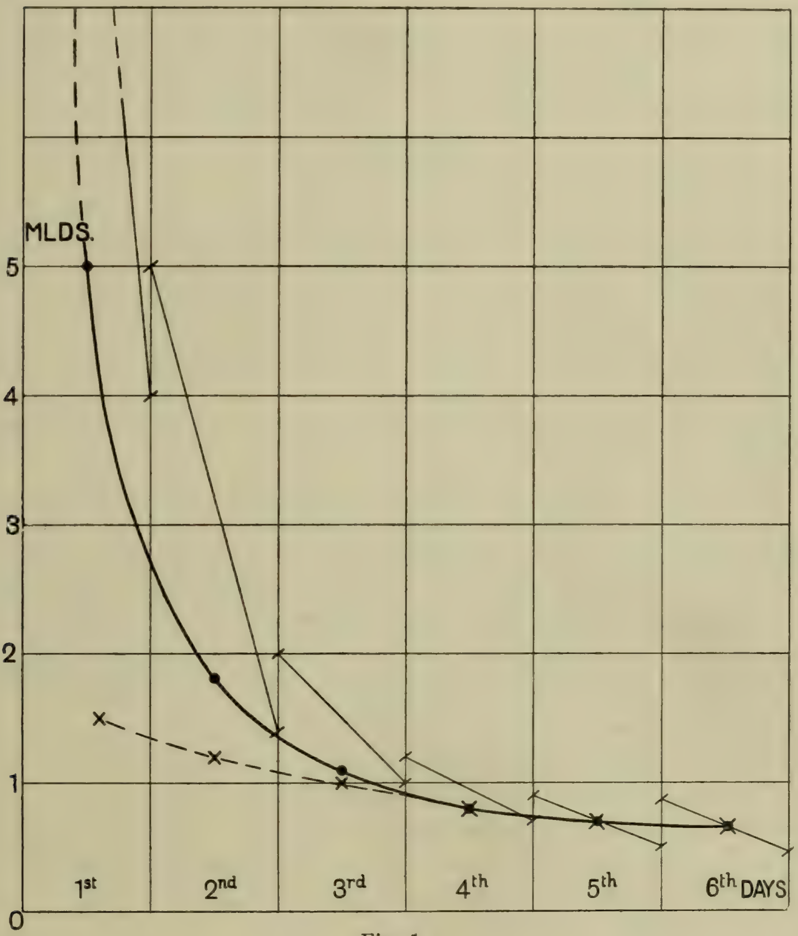

Fig. 1.

Thus the doses killing from the second to the sixth day give the products 3·6, 3·3, 3·2, 3·5 and 3·9. The corresponding products from Arrhenius and Madsen's relation are 2·4, 3, 3·48, 3·80, 3·92. The simple relation given, although probably not exactly of the best fitting type, which seems to be Lethal Time × $\sqrt[n]{\text{Lethal Dose}}$ = Constant, where n is a second constant, is likely to prove serviceable as a rough guide, giving results within the experimental error in the customary series of five guinea-pigs used in the standardisation of diphtheria toxins. It is certainly a useful mnemonic, for it states that the time of death is nearly inversely proportional to the dose of toxin. It seems to us to be doubtful whether any closer approximation is attainable under ordinary circumstances as regards the number of guinea-pigs likely to be used in standardisation. This is clearly shown by Table III for Toxin, No. 116, where the number of deaths are given and the days upon which death took place when various amounts of toxin were administered.

TABLE III.

Diphtheria Toxin No. 116 : *filtered* 28/1/01. *Tested* 1904.

Toxin in c.c.	No. of pigs	Time of death † in days								No. of survivals
		1	2	3	4	5	6	7	† days	
0·01	3	—	—	††	†	—	—	—	—	—
0·0075	1	—	†	—	—	—	—	—	—	—
0·005	4	—	†	††	—	†	—	—	—	—
0·004	3	—	—	†††	—	—	—	—	—	—
0·0033	5	—	—	—	†††	—	—	†	†30	—
0·0025	3	—	—	—	—	—	—	—	†10	2
0·0029	3	—	—	—	—	—	†	—	†24	1

It is noteworthy that a definite lethal amount of toxin cannot be relied upon to kill on any particular day, but the probability is that it will kill on one of two days immediately succeeding each other. This table seems to us to be typical of the usual experience met with in the determination of minimal lethal doses, for even in this case, where 22 guinea-pigs were used as test animals, the lethal dose 0·005 c.c. could not be relied upon to kill with absolute certainty on the third day, nor did 0·0033 c.c. give a lethal effect always on the fourth.

Further, the examination of the above-mentioned 200 brews of diphtheria toxin demonstrates conclusively that the number of animals dying on the seventh day to about the twenty-first day is so small and the irregularities so great as to render any calculation of a lethal dose for this period of no practical value.

Lethal Effect of Toxin-Antitoxin Mixtures.

As the L + dose of toxin denotes that quantity of toxic fluid which neutralises the unit of antitoxin, I.E., and still gives a minimal lethal dose in excess, it is evident that when the time limit for the L + dose is varied from the second to the sixth day the relative values will approximate more closely than we found to be the case for lethal doses of toxic fluid alone. This can be seen from Tables IV and V. In Table IV the range of L + dose killing on any day after inoculation is given from the year 1901 to 1905 for the best investigated toxin, viz. No. 116. The average values are shown in the last series, and taking the L + dose for the third day as unity the relative values for death on the other days are indicated in Table V. From Table V it may be seen that the range of dose for any particular day is considerable, but the "scatter" of the data is such that the mean values have an experimental error of ± 2% at the outside.

TABLE IV[1].

Toxin No. 116 *in dilution* 1 : 5, *L + in c.c.*

Time of death in days					
2	3	4	5	6	Date
·6	·61	·63	·61	·60	April—Sept. 1901
·65	·75	·70	·62	—	
(4)	(8)	(3)	(3)	(1)	
·72	·70	·64	·74	·66	Oct.—Oct. 1901–2
·78	·76	·70	—	·68	
(13)	(14)	(2)	(1)	(2)	
·68	·68	·68	·70	·68	Oct.—Oct. 1902–3
·78	·74	·74	—	—	
(14)	(8)	(5)	(1)	(1)	
·70	·68	·68	·66	·68	Oct.—Oct. 1903–4
·74	·76	·72	·74	·70	
(3)	(10)	(4)	(3)	(3)	
·70	·68	—	·68	—	Oct.—Feb. 1904–5
·74	·74	—	—	—	
(3)	(8)	—	—	—	
·73	·72	·70	·70	·68	1901–5

TABLE V.

Lethal time. Days	L+ doses corresponding		
	No. 116	No. 58	Mean
2	1·015	1·02	1·017
3	1·00	1·00	1·000
4	0·97	1·00	0·985
5	0·97	0·97	0·970
6	0·95	0·97	0·960

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[1] Bracketed figures indicate number of test animals.

A very similar relation was obtained for another toxin, No. 58, as is indicated in Table V. Taking the mean of these observations, we find that the L + doses and corresponding lethal times show a straight line relationship.

It follows also from Table IV that the neutralising power of the toxin No. 116 did not change appreciably during the period October, 1901, to February, 1905, although during this period the minimum lethal dose had increased by 100%, viz. 0·002 c.c. to 0·004 c.c. This is confirmatory of Ehrlich's view that on standing the toxic property of diphtheria toxin diminishes, whereas its neutralising power is, within the experimental error, unchanged. From April, 1904, onwards the toxicity of No. 116 appears to have remained constant, and the curve representing the decrement with time of standing is probably of hyperbolic form and similar to those found by Madsen for the disappearance of antitoxin from the animal system, the deterioration of tetanolysin, etc. A corresponding diminution in toxicity and constancy of neutralising power was observed for toxins Nos. 58, 27, 136 and 198.

The influence of the variation in the susceptibility of the test animals is also markedly shown on injecting mixtures of toxin-antitoxin. Table VI gives a summary of the results obtained when

TABLE VI.

Lethal Effect of Toxin-Antitoxin Mixtures.

Toxin No. 116 in dilution 1 : 5 + 1 I.E.

Toxin c.c.	Pigs No.	Days						Survivors	
		1	2	3	4	5	6	No.	%
1·2 —0·8	10	6	4	0	0	0	0	0	0
0·8 —0·75	15	1	9	4	0	0	0	1	6·6
0·75—0·7	33	0	10	15	2	1	0	5	15
0·7 —0·65	20	0	2	7	3	0	2	6	30
0·65—0·6	32	0	5	4	2	3	1	17	53
0·6 —0·5	16	0	1	0	0	0	1	14	87
0·5 —0·4	10	0	0	0	0	0	0	10	100
0·4 —0·3	8	0	0	0	0	0	0	8	100
0·3 —0·2	5	0	0	0	0	0	1	4	80

different amounts of toxin were used, the fluid containing unit antitoxin, *i.e.* 1 I.E., in each case. For example, with doses between 0·7 and 0·75 c.c. of a 1 in 5 dilution of toxin No. 116, out of 33 guinea-pigs, 10 died on the second day, 15 on the third, 2 on the fourth, 1 on the fifth, 0 on the first and sixth, while 5 pigs, or 15% of the whole number, survived the sixth day. It seems to be clear from this table, which we regard as very typical of the phenomena met with in investigating L + doses, that the determination of the dose corresponding to any particular lethal time must be the result of the interpolation of a large number of experiments, and that any relation found to exist between lethal dose and lethal time can form, at the best, only a rough guide in the estimation of free toxin in mixtures of toxin and antitoxin.

Loss of Weight and Lethal Time.

An endeavour was made to control the relations deduced by Arrhenius and Madsen between the variation in weight of the test animals and the time elapsing after inoculation, all the series of Table VI for toxin No. 116 being examined and mean values obtained for loss of weight on any particular day from the first to the fourteenth. The first and most striking result was that, although guinea-pigs which recovered showed at some period a maximum loss of weight, this maximum did not occur for all doses on the fifth day. Further, the shapes of the curves connecting lethal time and loss of weight were not similar to that which represents Arrhenius and Madsen's ratios (see Fig. 2). Quite other relations seem to exist; in the first place, non-lethal doses do not depress the weight of the test animals as a rule on the first day, but the weight continues to rise, and, with the smaller doses, at quite a normal rate, as will be seen from a subsequent paper. With larger doses the weight at the end of the first day may be constant, slightly increased or slightly depressed. Only with large lethal doses, in general, is a marked depression obtainable. Secondly, the injection of sub-lethal doses, in which the antitoxin is in great excess, leads in many cases to a marked temporary depression on the first day. This we attribute to "shock," for it has been observed that on the subcutaneous injection of perfectly innocuous fluids, such as normal saline, distilled water, distillates of broth, etc., a similar decrement of weight took place during the 24 hours succeeding inoculation. The mean values of a large number of tests show, however, that the relation first mentioned holds in general, viz., that after the injection of all mixtures of diphtheria toxin and antitoxin the animal continues to increase in weight, but that with increasing amounts of free toxin this increment in weight is more rapidly followed by a decrement, and with non-lethal doses the maximum weight of the pig is found at the end of 24 hours. It appears to us that these maximal weights would, if they were ascertained either by direct determination or by interpolation from a few observations, form more reasonable origins for the weight-time curves corresponding to different doses. As an approximation, especially in view of the unreliability of the results obtained during the first 24 hours, we decided to take the weight at the end of the first day as the origin of each curve, or, in other words, as the initial weight of the test animal. On pursuing this course several relations, formerly obscured, became immediately apparent, and these may be gathered from Fig. 2, curves 1 to 6. For pigs which recovered no maximum loss in weight occurred for doses of 0·4 c.c. to 0·3 c.c. of toxin No. 116 corresponding to the eighth series of Table VI, as shown in Fig. 2, curve 6. Series 7, Table VI, gave curve 5 in the same figure, and indicates that with 0·5 c.c. to 0·4 c.c. of the diluted toxin the maximum loss of weight took place on the third day. Curve 4, belonging to series 6, Table VI, gives a maximum loss on the fourth day for 0·6 c.c. to 0·5 c.c. of the toxic fluid. For series 5 no definite maximum loss could be obtained, and, indeed, the form of curve 3 seems to indicate that this is a transition relation between those holding for pigs which recover and others applicable to pigs which die within the first few days. The general downward trend of the curve is probably due to the fact that only $53\,^0/_0$ of the test animals survived the sixth day

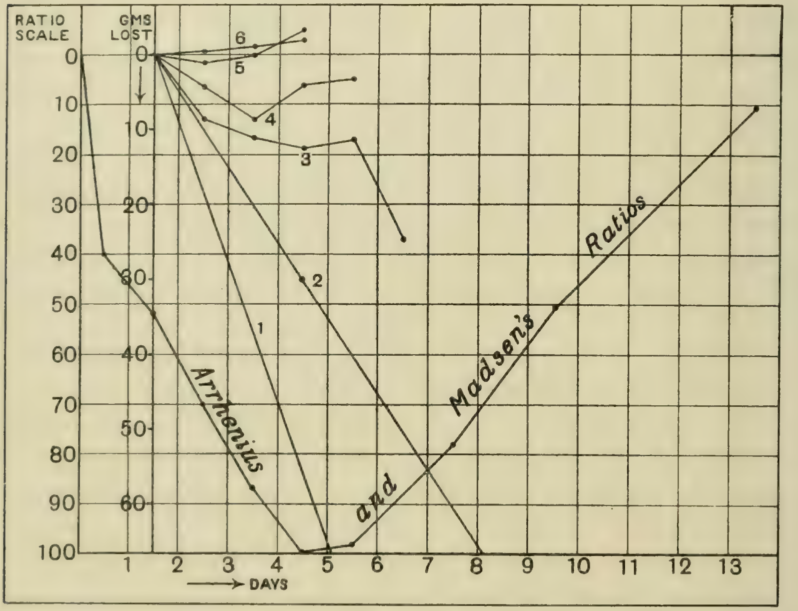

Fig. 2.

on the injection of 0·6 c.c. to 0·65 c.c. of toxic fluid. The remaining curves, 2 and 1, show in general the time-weight relations existing for amounts of toxin which can be more accurately described as lethal doses or M.L.DS. and L + doses when antitoxin was present, and represent in the latter case the effects of amounts of toxin No. 116 varying from 0·65 c.c. to 0·8 c.c. of the dilution stated in Table VI. In this lethal zone, enclosed by the two curves now under consideration, the injection of 0·65 c.c. gives rise to curve 2 and increasing quantities of toxin give curves which incline more steeply to the abscissa, until finally with 0·8 c.c. the position occupied by curve 1 is reached.

With doses greater than 0·8 c.c. up to 1·2 c.c. the inclination does not appreciably exceed that of curve 1, and as the values obtained from these doses refer chiefly to pigs which died within 24 hours they have been omitted. The lethal rays thus obtained for the time-weight relations of doses between 0·65 c.c. and 0·8 c.c. are, within the experimental error, straight line relations, and it will be seen from a subsequent paper that they practically correspond to the "starvation curve" of the guinea-pig under normal conditions.

Summary of Conclusions.

1. The relation between lethal doses of diphtheria toxin and the times in which they kill guinea-pigs, or lethal times, is approximately such that the lethal dose multiplied by the corresponding lethal time gives a constant value—a hyperbolic relation.

2. Deductions with regard to lethal doses from deaths occurring on the first day or on any day after the sixth give such widely divergent results that, in our opinion, they are at present of negligible value.

3. Under the ordinary circumstances of standardisation of diphtheria toxin and antitoxin the relation given in conclusion No. 1 is as close an approximation as any alternative likely to prove of practical utility.

4. The graphic representation of L + doses against lethal times gives a straight line relationship.

5. Five of the best investigated toxins confirmed Ehrlich's views in so far as that with toxicities diminishing by 50 % their neutralising powers remained practically unimpaired.

6. The individual sensitivities of guinea-pigs to free diphtheria toxin render any general relation between lethal times and doses of little value when a small number, *e.g.* 5, of test animals is inoculated.

7. Even with a dose of toxin which would, in any ordinary series of tests, be regarded as an L+ dose, 5 out of 33 guinea-pigs survived the sixth day or 15%[1], and the M.L.D. of a well-investigated toxin showed similar variations; consequently both the L+ and M.L.D. values of a toxin are not those amounts which kill with certainty in a fixed time, but those which will cause death in that time with the greatest probability.

8. After inoculation, guinea-pigs unless suffering from the consequent "shock," continue to increase in weight throughout the first day, even with doses that may subsequently prove lethal, and with highly toxic doses the variation in weight during this time is of little significance.

9. The greater the amount of free toxin injected, the more rapid is the increment in weight followed by a decrement.

10. The weight of a test animal at the end of 24 hours after inoculation forms a better normal or origin with which to compare the subsequent time-weight relations than the initial weight before injection.

11. On the basis of Conclusion 10 the general connection is found that with increasing amounts of free toxin the position of the maximum loss of weight is gradually transferred from the second to the fifth day for guinea-pigs which survive over six days.

12. The time-weight ratios for almost certainly lethal doses give straight line connections, corresponding probably to starvation curves of the guinea-pig.

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[1] In the standardisation of diphtheria antitoxin at the Lister Institute, Elstree, 0·72 c.c. of toxin No. 116 was considered to represent the L+ dose for the fourth or fifth day instead of the third, which, according to the above calculations, would harmonise best with the mean of all the observations. The fluids so standardised had then at least the value ascribed to them and were probably higher in content of antitoxin.

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A CONTRIBUTION TO THE BACTERIOLOGY OF POST-SCARLATINAL DIPHTHERIA.

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The basis of this paper is the analysis of the bacteriological examination of the fauces of a series of 1019 patients admitted to hospital as cases of scarlatina and a consideration of their subsequent clinical histories. A practice was made of swabbing the fauces of scarlet fever cases without any selection on their admission to hospital. In all cases one bacteriological examination was made and in a number of the cases the examination was repeated.

Method of carrying out the Examination and Criteria employed for determining the presence of Bacillus diphtheriae.

The fauces were swabbed with a sterile cotton-wool swab while the patient was still in the receiving room, so as to obviate any possibility of infection in the hospital. The swab was immediately rubbed well on the surface of a solidified blood serum tube. The tube was incubated at 37° C. and examined after 18 hours. Films were made and stained with Löffler's methylene blue, and in those cases in which there was a doubt as to the character of an organism resembling the diphtheria bacillus separate films were made and stained by Gram's method, by Neisser's stain and by toluidene blue. With this last stain true *B. diphtheriae* stain, light blue, the granules being a deep purple and very definitely outlined. Diphtheroid organisms show polar staining of much less definite character—in most cases staining almost uniformly.

Results of the Examinations.

Of the 1019 cases the throat was examined in 1017 and the nose in 165. The examination of the nose was omitted in the majority of the cases. It is recognised that the results would have been more valuable had such an examination been made throughout the whole series.

Of the total 1019, *B. diphtheriae* were found in 75 cases and diphtheroid organisms in 12. That is to say 7·36 % of cases of scarlet fever examined on admission showed the presence of *B. diphtheriae* in the nose or throat on one examination and 1·07 % showed diphtheroid organisms (*B. Hofmanni*).

These occurred thus.

In the throat 1017 cases examined:

59 or 5·8 % showed *B. diphtheriae.*
7 or ·68 % showed diphtheroid bacilli.

In the nose 165 cases examined:

21 or 12·72 % showed *B. diphtheriae.*
8 or 4·84 % showed diphtheroid bacilli.

Comparative results are available. In two of the other hospitals of the Metropolitan Asylums Board the following results were obtained.

In the throat: (*a*) One series of 203 cases. 5·41 % with diphtheria bacilli and 1·2 % with diphtheroid[1].

(*b*) A series of 87 cases. 6·8 % with diphtheria bacilli[2].

In the nose: (*a*) A series of 202 cases. 12·37 % with diphtheria bacilli and 53·7 % with diphtheroid[1].

(*b*) Lambert Lack (1898), in a series of 100 cases in the out-patient department of a large general hospital, found diphtheria bacilli in 13 % of the noses examined and diphtheroid organisms in 52 %[3].

These results agree closely with those obtained in the present series as far as true diphtheria bacilli are concerned, but the presence of diphtheroid organisms in more than half the noses examined is not in agreement with the results obtained during the present investigation. This discrepancy is probably due to a wider interpretation by these writers of the term "diphtheroid." The term diphtheroid in this paper refers to the class of micro-organisms usually known as Hofmann's bacillus.

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[1] *Report Metrop. Asylums Board*, 1900.

[2] *Trans. Epid. Soc. London*, vol. xv.

[3] *Med. Chir. Trans.*, vol. LXXXII.

That is to say, then, that of the 1019 cases 75, or 7·36% were harbouring the diphtheria bacilli and 12 or 1·07% other organisms, presumably (in the absence of further bacteriological examination) of the same type.

932 cases were free from such organisms as far as could be seen in one examination.

Subsequent History of the Cases Examined.

Of the 932 cases six developed clinical diphtheria.

Five of the faucial type on the 9th, 22nd, 46th, 48th and 50th days of scarlet fever.

One of the laryngeal type on the 38th day—this required tracheotomy but recovered.

These six cases, or ·64% of "bacilli free" cases, were presumably cases of hospital-contracted diphtheria.

Of the 75 cases which showed the presence of diphtheria bacilli on admission four cases, or 5·3%, developed clinical diphtheria, three cases occurring on the 4th, 5th and 31st days—the last one of which died of diphtheria. The fourth case is interesting in that it showed diphtheria bacilli in the nose only and not in the throat. It developed typical faucial diphtheria on the 7th day.

There were three other cases which are worth mentioning.

(*a*) Diphtheria bacilli in the nose only on admission—on the 31st day there were diphtheria bacilli in the nose and throat—on the 44th day pharyngitis occurred, with no formation of membrane.

(*b*) This case was certified as diphtheria but was diagnosed scarlet fever on admission and showed diphtheria bacilli in the nose only. There was some paralysis of the soft palate on 44th day.

(*c*) Diphtheria bacilli in the throat only on admission—the patient developed tonsilitis with no membrane on the 26th day.

The throats of these 75 cases presented on admission no evidence on examination of the presence of the bacilli—they differed in no way from the ordinary variety of throat conditions seen in scarlet fever.

Of those cases, then, which showed no bacilli on admission ·64% contracted diphtheria, while of those which had already diphtheria bacilli in the throat or nose 5·3% contracted diphtheria. This may be interpreted as indicating that many of those cases of post-scarlatinal diphtheria apparently contracted in hospital are not really cases of hospital infection, but are due to an attack on the organism by bacilli already present—the scarlet fever having possibly produced a condition of lowered resistance. It is, further, interesting to note that the cases which had diphtheria bacilli on admission developed the clinical manifestations on the 4th, 5th, 7th and 31st days of scarlet fever, while those which were "bacilli free" showed clinical diphtheria at later stages, viz. 9th, 22nd, 46th, 48th and 50th days. Whether this interpretation be accepted or not, the results are sufficiently striking to justify the routine bacteriological examination of every case of scarlet fever admitted and the continuance, in those cases in which bacilli are present, of routine throat treatment well into the stage of convalescence and even right up to the time of discharge from hospital. That one case should have bacilli in the nose only and yet develop faucial diphtheria supports the routine examination of the nose as well as of the throat.

It is striking that of the 75 cases which had diphtheria bacilli on admission only four, or 5·3% (or if we include the three doubtful cases mentioned above seven, or 9·3%) developed clinical diphtheria. That is to say that the possible effect of scarlet fever in raising the virulence of the diphtheria bacilli (or lowering the resistance of the patient) was only seen at the outside in 9·3% and only definitely in 5·3% of the patients in which all the conditions for such a result were already present.

Of the 12 cases which showed Hofmann's bacilli on admission four showed these organisms in the throat only; five in the nose only; and three in both throat and nose—in none of these cases was there any clinical manifestation that could be associated with their presence, nor were any true diphtheria bacilli found in these cases on repeated examinations.

Persistence of the Bacilli.

In 29 cases repeated examinations were made to determine how long the bacilli remained in the throat while daily treatment of the throat was being carried out with antiseptic lotions. The periods during which the bacilli remained present were in days: 2, 2, 2, 2, 2, 3, 3, 4, 5, 9, 9, 11, 11, 15, 16, 16, 17, 18, 19, 22, 23, 23, 26, 28, 31, 32, 33, 34, 43. In none of these cases did there develop any clinical evidence of diphtheria. In 11 cases the bacilli disappeared under treatment before the 10th day, and in 19 cases before the 20th. In six other cases, however, the bacilli were still present after 16th, 26th, 38th, 43rd, 45th and 122nd days. In these cases it was not possible, because of the transfer of the patients to a convalescent hospital, to continue the examination to determine the time of disappearance of the bacilli. In only one of the six were there any clinical signs, that is the one in which they were still present after 43 days—this was the same case referred to above, in which tonsilitis occurred on the 26th day.

The conclusion is, then, that in most of these cases the bacilli quickly disappear under treatment, but that in other cases they may remain long periods, in one case so long as 122 days.

There is still another aspect to be considered. That is that of the 932 cases which showed no diphtheria bacilli on admission there was a total of 28 cases (including the six cases of clinical diphtheria) which subsequently showed the presence of such bacilli.

These 28 cases can be sub-divided thus:

A. Nineteen cases in which the nose was not examined but no diphtheria bacilli were present in the throat on admission.

(i) In 5 cases diphtheria bacilli were found associated with purulent rhinorrhoea on the 8th, 35th, 41st, 44th and 62nd days of scarlet fever.

(ii) In 11 cases diphtheria bacilli were found in the throat, five cases with no clinical signs but bacilli found on the 2nd, 3rd, 13th, 14th and 43rd days.

Six cases, as above with clinical diphtheria on the 9th, 22nd, 38th, 46th, 48th and 50th days of scarlet fever.

B. Nine cases which showed no diphtheria bacilli in nose or throat on admission.

(i) Two of these showed bacilli in both nose and throat on the 30th and 61st days of scarlet fever respectively.

(ii) Five showed bacilli in the nose only on the 34th, 42nd, 45th, 48th and 51st days—all of these had purulent rhinorrhoea but no ulceration or membrane on the septum or the turbinates.

(iii) Two showed bacilli in the throat only on the 29th and 61st days respectively—the latter of these showed the persistence of the bacilli in the ear discharge till the 139th day.

It may be objected that the comparison above of 5·3% of clinical diphtheria cases in which bacilli were present on admission with ·64% of apparently hospital-contracted clinical diphtheria is not a just one, but that all bacteriological infection, whether followed by clinical diphtheria or not, should be regarded as of hospital origin.

Even if this is done and the whole 28 cases included it makes 2·75% as against 5·3%, and there is abundance of reliable authority for assuming that the bacilli were probably overlooked on the first examination, and in 19 of the cases it is possible that the bacilli were present in the nose.

Conclusions.

The most interesting facts brought out by this investigation are:

1. That of cases of post-scarlatinal diphtheria with clinical signs the percentage of those which come into hospital with bacilli already present is more than eight times as high as the percentage of those which are presumably infected in hospital.

2. That while in the majority of the cases which are harbouring diphtheria bacilli on admission, the bacilli rapidly disappear under treatment yet, in a certain small percentage, the bacilli may persist for a long time, though not necessarily producing clinical manifestations.

3. That of those cases which had diphtheria bacilli present on admission less than 10% (on the widest calculation) developed clinical signs and only 5·3% developed typical diphtheria.

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THE RELATIVE FREQUENCY OF VARIOUS TYPES OF STREPTOCOCCI IN SCARLATINA.

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The Streptococci obtained in Scarlet Fever.

It has long been well established that during the height of scarlet fever streptococci flourish and become greatly predominant in the pharyngeal and buccal mucus. To quote only a few of the many recorded observations.

Lemoine (1895) in the mucus from 117 throats in scarlet fever found streptococci alone 93 times and with other organisms in 14.

Baginsky and Sommerfeldt (1900) in 701 cases found that streptococci were absent only five times.

The latest results are those obtained by Ruediger (1906). He inoculated (with broth in which the swab used to collect the material had been washed) blood-agar tubes, from which he made plates. On the medium the streptococci grew as grey colonies surrounded by a clear area of haemolysis. Pneumococci grew as green colonies and could thus be distinguished from streptococci. The colonies obtained were picked off and confirmed by further morphological and cultural investigation. Of 75 throats thus examined during scarlet fever, 2 showed only streptococci, 31 showed 60—95% of streptococcal colonies, 21 showed 15—40% streptococci, and 20 showed streptococci and pneumococci.

Ruediger makes this statement. "As a rule the streptococcus colonies greatly predominate over the other colonies when the inflammation of the throat in scarlet fever is pronounced, and they rapidly decrease in number with the subsidence of the throat symptoms....When the throat symptoms are mild the proportion of streptococcal colonies to other colonies is quite small."

Ruediger further gives the results of his examination of 51 normal throats. "Streptococci were present in 30 out of 51 cases examined but were never present in large numbers and were entirely absent in 21 cases." The fact of this almost invariable preponderance of streptococci in the throats of early cases of scarlet fever agrees with the writer's experience. In over 250 plates made and examined the streptococci were present in nearly every case in much greater numbers than all the other types of colonies together.

Assuming the fact of an increase in the number of streptococci taking place in the throat during the first few days of scarlet fever as having been demonstrated, the object of this enquiry was an attempt to discover whether these streptococci showed any uniformity in type.

For this purpose the organic substances introduced by Gordon (1905) were employed and the streptococci were grouped according to the results produced by their growth on these media. The formula of the media used was peptone water 75 %, beef broth (made from bovine heart muscle) 25 %, and in this was dissolved 1 % of the test substance.

The streptococci examined included those obtained from abscesses, from the nasal discharge and from the tonsils during scarlet fever. The pus was obtained from the abscesses by inserting a sterile hypodermic needle after sterilizing the skin and removing a few drops of pus, which were grown for 18 hours in broth and then plated off on agar in three dilutions of the broth.

In order to reduce to a minimum the amount of contamination by the ordinary salivary organisms when obtaining specimens from the throat the mouth was first swabbed as dry as possible with cotton-wool. All debris and secretion were then rubbed off the surface of the tonsil with a sterile cotton-wool swab until the mucous membrane just began to bleed. Then a second sterile swab was at once used and soaked in the tonsillar secretion. This swab was then lightly rubbed over the surface of an agar plate. A glass rod bent at right angles was then sterilized and rubbed over this plate, then on to a second plate and straight on to a third. The result on the third plate was a very limited number of colonies (in most cases entirely streptococcal) which could be picked off and examined. From three to five single colonies were taken from each plate and grown separately in broth for 48 hours at 37° C. At the end of 48 hours films were made and examined under the microscope. Any that did not show a uniform morphological type, or, at least, nearly uniform, were discarded. Then from the broth culture the various chemical media were inoculated. In all 19 strains were examined from abscesses, 80 strains from throats, 1 strain from a case of scarlet fever post-mortem, and 1 from a case of rhinorrhoea within the first day after the onset of the discharge.

These 101 different specimens of streptococci gave the following results with the above tests:

(*a*) The whole series failed to reduce neutral-red after three days' growth at 37° C. under strictly anaerobic conditions.

(*b*) Every member of the series grew on gelatin in two days at 22° C.—none liquefied the gelatin, but the amount of growth shown in two days varied within moderate limits.

(*c*) As a general rule—with only a few exceptions—the streptococci fermented saccharose and lactose.

The remaining reactions need to be considered more in detail.

To take first those streptococci obtained from abscesses. These represent probably the streptococci which invade the general circulation and which, any serum which aims at the improvement of "septic" symptoms in scarlet fever, must attack.

The 19 strains present four distinct types.

No. of specimens	Neutral red	Saccharose	Lactose	Raffinose	Inulin	Salicin	Mannite	Milk	Gelatin at 22° C.
13	–	+	+	–	–	+	–	A	+
4	–	+	–	–	–	+	–	–	+
1	–	+	+	–	–	+	+	A	+
1	–	+	+	–	–	+	–	A. C.	+

A. C. means the production of acidity and clotting in litmus milk.

A means acidity only.

+ = the production of acidity except in the case of gelatin where it means simply "growth."

It will be seen then that the predominant type of streptococcus found in the abscesses examined was one which fermented saccharose, lactose and salicin, which produced acidity but no clotting in milk and which left the other media unaffected. In all the media growth was quite vigorous. These abscesses occurred between the 8th and 34th days of disease. The fact that 19 specimens obtained from abscesses could be divided into four distinct types suggests that if favourable results are to be obtained from serum therapy it will be by the use of a polyvalent serum.

Rhinorrhoea.

One strain fermented lactose, saccharose, salicin and mannite and produced only acidity in milk.

Streptococci from Throats.

The following table includes all those strains of streptococci obtained by the method described from the throat in scarlet fever.

TABLE A.

Neutral red	Saccharose	Lactose	Raffinose	Inulin	Salicin	Mannite	Milk	Gelatin	No. of specimens
−	+	+	−	−	+	−	A	+	40
−	+	+	−	−	+	+	A	+	13
−	+	+	F	−	+	−	A	+	2
−	+	+	+	+	+	−	A	+	1
−	−	+	−	−	+	+	A	+	1
Fermented Glucose only									2
−	+	+	−	+	−	−	A	+	1
−	+	+	−	−	−	−	A	+	1

TABLE B.

−	+	+	−	−	+	−	A. C.	+	6
−	+	+	−	−	−	−	A. C.	+	5
−	+	+	+	−	−	−	A. C.	+	4
−	+	+	+	+	+	+	A. C.	+	1
−	+	+	+	+	+	−	A. C.	+	1
−	+	+	−	F	+	−	A. C.	+	2

In Table A are included all those that do not clot milk but produce only acidity. In Table B are included those that both produce acidity and clot the milk. In both tables the letter F is intended to indicate that a faintly acid reaction was produced after five days.

(These 80 streptococci from the throats were isolated from 25 patients between the second and ninth days of disease with one exception, which was obtained on the 26th day of the disease. The colonies were picked off quite indiscriminately from the original plates.)

Streptococcus obtained Post-mortem.

This strain was obtained from a case of scarlet fever which was typically of the "Toxic" type. Death occurred on the third day of disease, a rigor having taken place about six hours before death.

Cultures were made from the blood removed from the left ventricle in a sterile pipette, from the splenic pulp and from the liver. Two sets of blood cultures were taken from the heart—both remained sterile. The culture from the liver showed a mixed staphylococcus and *Bacillus coli* culture. The spleen showed a pure streptococcal culture. This streptococcal culture was plated out on agar and single colonies examined. Morphologically it was of "medius" type—it grew in broth with a sandy sediment (type B)[1]—grew fairly well on gelatin at 22° C. in two days, produced acidity but no clotting in milk, and fermented saccharose, lactose, salicin and mannite, but showed no reduction of neutral-red.

Its pathogenicity was tested on mice and it produced a local abscess at the site of injection in four days and death of the mouse on the seventh day. The streptococci could not be recovered from the heart or liver, but only from the abscess of the mouse.

To compare, then, the results obtained by these fermentation tests on the various streptococci. First let us take the two types shown below, which between them include the great majority of the specimens examined. It will be seen that they are closely similar, the differential test being the power of fermenting mannite.

	No. of specimens	Neutral red	Saccharose	Lactose	Raffinose	Inulin	Salicin	Mannite	Milk	Gelatin
Abscess	13	−	+	+	−	−	+	−	A	+
Throat	40	−	+	+	−	−	+	−	A	+
Abscess	1	−	+	+	−	−	+	+	A	+
Throat	13	−	+	+	−	−	+	+	A	+
Rhinorrhoea	1	−	+	+	−	−	+	+	A	+
Cadaver	1	−	+	+	−	−	+	+	A	+

The fact that not one of these 101 cases reduced neutral-red is striking, several controls were made with streptococci from normal saliva on the same neutral-red media and they produced a reduction.

Gordon (1905) describes five samples of streptococci obtained by him from scarlet fever cases—three of these gave positive reactions to saccharose, lactose and salicin only, and two gave positive reactions to the same three reagents as well as to mannite. Thus all the five specimens belong to the same two groups as the majority of the strains in this series.

Andrewes and Horder (1906) have collected 1200 specimens of streptococci subjected to these differential tests. Thirty-three of these strains were obtained from scarlet fever cases, and these fall under the classification used by those authors as follows:

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[1] *Vide infra.*

S. pyogenes	12
S. salivarius	1
S. anginosus	20

The *S. pyogenes* group corresponds with that into which the large majority of the cases in this series fall. Of the 20 strains included in the *S. anginosus* group, nine do not reduce neutral-red and the reactions given by these nine correspond closely with the reactions given by members of the present series.

A careful repetition of the nine tests, in many of the cases after long intervals—in some cases three months—corroborated the assertion of these other writers that each strain of streptococcus preserves its characteristics unchanged.

Culture appearances in Broth.

In each case a note was kept of the way the streptococcus grew in broth.

The types of growth are divided into four.

Type A. Uniform turbidity with little deposit.
B. Clear broth with sandy deposit.
C. Clear broth with small flocculent masses adhering to the bottom and sides of the tube.
D. Clear broth with larger floccular masses on the bottom of the tube.

The following tables show the chemical reactions given by these different types.

It will be seen from these tables that there is no close connection between the type of growth in broth (the only method of separating streptococci before the introduction of these "nine tests") and the reactions given with the tests under discussion. It is interesting to

From Abscesses.

	Neutral red	Saccharose	Lactose	Raffinose	Inulin	Salicin	Mannite	Milk	Gelatin	No. of specimens
Type B	–	+	+	–	–	+	–	A. C.	+	1
	–	+	+	–	–	+	–	A	+	8
	–	+	–	–	–	+	–	–	+	4
Type C	–	+	+	–	–	+	–	A	+	5
	–	+	+	–	–	+	+	A	+	1

From other sources.

Type A.

Neutral red	Saccharose	Lactose	Raffinose	Inulin	Salicin	Mannite	Gelatin	Milk	No. of specimens
–	+	+	–	–	+	–	+	A. C.	2
–	+	+	–	–	–	–	+	A. C.	4
–	+	+	+	–	–	–	+	A. C.	3
–	+	+	+	+	+	+	+	A. C.	1
–	+	+	–	–	+	–	+	A	2
–	+	+	F	–	+	–	+	A	1
				Type B.					
–	+	+	–	–	–	–	+	A. C.	1
Glucose only							+		2
–	+	+	–	–	+	–	+	A	4
–	+	+	–	–	+	+	+	A	1
				Type C.					
–	+	+	–	–	+	–	+	A. C.	4
–	+	+	–	F	+	–	+	A. C.	2
–	+	+	–	–	+	–	+	A	30
–	+	+	–	–	+	+	+	A	14
–	+	+	F	–	+	–	+	A	2
				Five other varieties with one member each.					
				Type D.					
–	+	+	+	+	+	+	+	A. C.	1
–	+	+	–	–	+	–	+	A	4

note, however, that in the group, Type A, which so far as morphology is concerned coincides with *Streptococcus brevis,* 10 of the 13 members clotted milk, while in the group, Type C, morphologically *Streptococcus longus,* 51 of the 57 did not clot milk.

That is to say the majority of the streptococci found in scarlet fever throats correspond with the *S. longus* type.

This series of streptococci then shows:

(1) That 50°/₀ of the throat streptococci examined give identical results on these selected media.

(2) That 68·4°/₀ of the streptococci obtained from the abscesses give identical results.

(3) That the majority of throat streptococci in scarlet fever are of the same type as the majority of streptococci obtained from abscesses in various parts of the body.

(4) That if we include the specimens that "fermented" mannite, then we find that 69°/₀ of the streptococci obtained from throats,

abscesses, nose and cadaver in scarlet fever are of identical type and correspond with the specimens isolated by Dr Gordon from throats, abscesses, cadavers, ears and cervical glands in scarlet fever.

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An Experimental Enquiry into the Nature of the Substance in Serum which influences Phagocytosis.

(*Second Communication.*)

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(Communicated by Dr. C. J. Martin, F.R.S. Received April 23,—Read May 30, 1907.)

In a previous communication it was shown that in certain immune sera, *e.g.*, in anti-staphylococcus, in anti-dysentery, and in anti-typhoid serum, there are present thermostable substances which, by their action, prepare the homologous organisms for phagocytosis. The thermostability in the case of these immune sera could be demonstrated by the use of Wright and Douglas' method, whereas in the case of normal serum another method was, as a rule, necessary. In the case of the serum of the horse, however, it was pointed out that even by Wright and Douglas' method the increase of phagocytosis, observed by using normal heated serum, was, as compared with salt solution, generally considerable.

In view of certain experimental data and having regard to Ehrlich's theory, the conclusion was arrived at that the thermostable substances of normal and immune sera were probably identical.

It was further shown that, when submitted to a temperature of 60° C., both immune and normal serum suffered a rapid and sudden diminution in opsonic power. This change occurs in the first few minutes of heating, after which further loss appears to take place very slowly.

The results obtained suggested that the sensitising action of the serum might be due to the combined action of two substances, the one thermostable, the other thermolabile; the one of the nature of the "substance sensibilisatrice," the other of the nature of an alexin.

The present paper is an account of certain experiments carried out with the view of elucidating this point.*

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* In the course of writing this paper, a communication by Neufeld and Hüne has reached me, in which, in a criticism of my interpretation of these curves, they appear to have overlooked the fact that I had indicated the possibility that the sudden fall may be due to a destruction of complement. As at the time of the publication of my previous communication I had no experimental data to support that view, I preferred to leave the matter for further investigation. What I said was:

"Whether free complement may take part in the preparation of the microbe is difficult to determine. From the experiments detailed in this paper it is certain that it is not a necessary participant in this action. At the same time it is not improbable that the

THE INFLUENCE OF THE STRENGTH OF THE EMULSION UPON THE NUMBER OF ORGANISMS TAKEN UP BY THE LEUCOCYTES.

In Table I are shown the results obtained in two experiments carried out in the usual way by mixing one volume of serum, one volume of washed blood corpuscles and one volume of bacterial emulsion, putting the pipettes containing the mixtures in the incubator at 35°·5 C. for 15 minutes, after which the preparations for microscopical examination were made. The number of phagocytosed bacteria were subsequently counted, and the average number per leucocyte taken as the phagocytic index. As a rule, the number of organisms in 50 to 80 leucocytes were counted, and in many of the experiments the mean of two or three such counts by independent observers was taken. The emulsion of *Staphylococcus pyogenes aureus* was employed in different concentrations, full strength, half strength, quarter strength, one-eighth strength, and one-sixteenth strength.

Table I.

Emulsion of cocci concentration.	Average number of cocci phagocytosed in the case of—	
	Thick emulsion.	Thin emulsion.
1	86·0	16·5
$\frac{1}{2}$	52·5	9·6
$\frac{1}{4}$	17·4	6·4
$\frac{1}{8}$	7·9	2·9
$\frac{1}{16}$	3·0	1·3

The number of organisms taken up is, within the error of experiment, directly proportional to the concentration of the bacterial emulsion employed.

INFLUENCE OF DILUTION OF THE SERUM UPON THE NUMBER OF ORGANISMS TAKEN UP BY THE LEUCOCYTES.

When in similar experiments different concentrations of the same normal serum are employed, no direct proportionality can be demonstrated to exist between the serum concentration and the number of microbes phagocytosed. For example, the number of ingested organisms may be as great, or even greater, with the half, quarter, or even the one-eighth concentration, as with the full

immune body, when aided by complement, may act more powerfully, and that the sudden fall in the "opsonic" power of both normal and immune serum, on heating, is due to the destruction of the complement. I may revert to this subject on another occasion."

strength serum. This appears in a table in Wright and Douglas' (1903) paper, but no remark is made on the significance of the phenomenon.

The normal serum of several species of animal tested behaved in a similar manner in respect to dilution, as appears in Table II.

Table II.—Effect of Dilution of Normal Serum of Different Species. The Numbers indicate the Average Number of Micro-organisms taken up per Leucocyte.

Serum	Human serum and leucocytes.	Horse's serum and leucocytes.	Pig's serum and leucocytes.	Rabbit's serum and human leucocytes.	Human serum and leucocytes.
Micro-organism	Staphylococcus.	Staphylococcus.	Staphylococcus.	Staphylococcus.	Tubercle bacillus.
Concentration of serum—					
1	9·7	23·7	12·7	16·4	8·3
$\frac{1}{2}$	9·6	28·5	15·8	17·0	12·2
$\frac{1}{4}$	10·0	14·6	14·9	12·6	9·5
$\frac{1}{8}$	8·2	13·0	12·3	9·2	5·3
$\frac{1}{16}$	8·5	11·9	5·2	3·0	4·8
$\frac{1}{32}$	6·4	6·8	6·7	2·4	2·5
$\frac{1}{64}$	2·3	—	—	—	1·6

In addition to the experiments with staphylococcus, an experiment with the tubercle bacillus was also made, which shows that this relation is not limited to one micro-organism. Five dilution experiments on normal human serum, the diluent being normal salt solution, and the organism being the *Staphylococcus pyogenes aureus*, have been made.

The mean figures of these experiments for the pure serum, and the successive dilutions down to 1 in 32, if expressed in terms of unity are,

1, 1·16, 1·02, 0·71, 0·53, 0·35,

and the corresponding concentrations of serum,

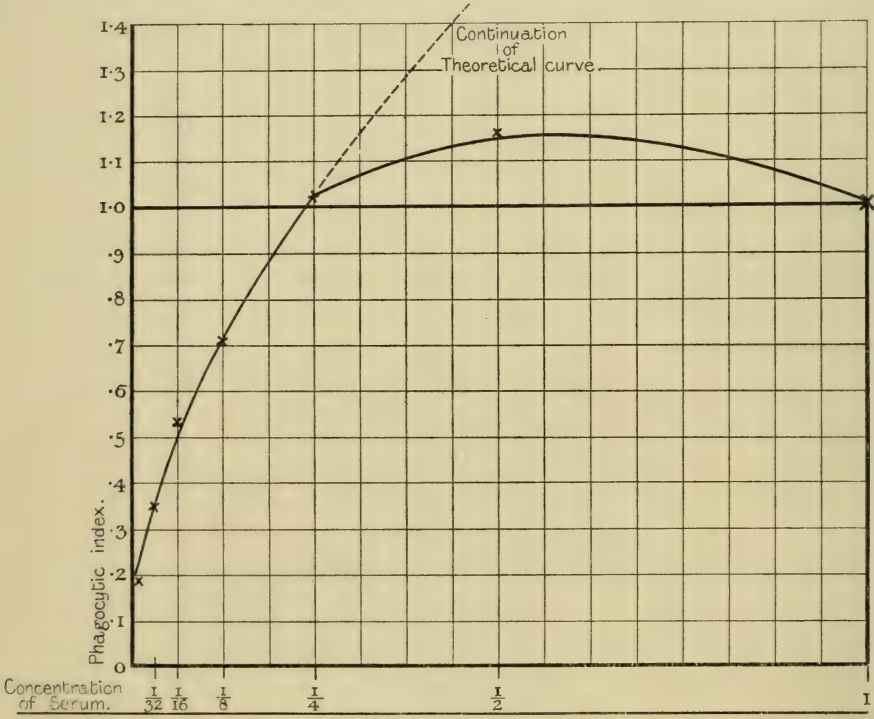
1, 0·5, 0·25, 0·125, 0·0625, 0·03125.

I am indebted to Dr. J. C. G. Ledingham for pointing out to me that after the second number there exists an approximate constant ratio between the second series and the squares of the first, as shown below:

$$
\begin{aligned}
(1{\cdot}02)^2 &= 4{\cdot}2\ \ (0{\cdot}25),\\
(0{\cdot}71)^2 &= 4{\cdot}03\ (0{\cdot}125),\\
(0{\cdot}53)^2 &= 4{\cdot}4\ \ (0{\cdot}0625),\\
(0{\cdot}35)^2 &= 3{\cdot}9\ \ (0{\cdot}03125),
\end{aligned}
$$

and that if the points (0·25, 1·02), (0·125, 0·71), (0·0625, 0·53), (0·03125, 0·35) are plotted, they are found to satisfy almost exactly the parabola whose equation is $y^2 = Kx$, where K = 4·1 (mean of 4·1, 4·03, 4·4, 3·9). The points (0·5, 1·16) and (1·1) obviously do not lie on the parabola.

The curve shown in the figure has been drawn from the mean of the figures for each dilution.



It will be seen that with the half concentration of serum there is a slight rise in the curve. This occurs so consistently that it can hardly be due to experimental error. From the quarter concentration onwards a fall is seen in the curve.

It is interesting to note that this square-root proportionality is analogous to what was found by Schutz (1885), Volhard (1901) and others working with albumin-pepsin and fat steapsine, etc. If these data are sufficiently accurate to be relied upon, it means that the phagocytosis is proportional to the square root of the dilution of serum for these concentrations, but that another factor is probably at work to account for the upper part of the curve.

The importance of this question of dilution in relation to the estimation of the amount of sensitising substances in the serum in normal, pathological, and immune conditions is obvious.

A striking instance may be given here. One specimen of an anti-staphylococcic horse serum, when diluted 32 times with saline, was found to have lost only 25 per cent. of its sensitising power, whereas the corresponding dilution of a normal serum of the same age had lost 90 per cent.

This subject in its various aspects will be dealt with more fully upon a future occasion.*†

THE INFLUENCE OF THE ADDITION OF FRESH SERUM UPON THE OPSONIC POWER OF HEATED IMMUNE SERUM.

As was pointed out in my previous communication, thermostable immune substance is capable of sensitising the microbes for phagocytosis in the absence of free complement. Metchnikoff's view, that in such cases the complement may be supplied by the leucocytes, is a possible interpretation, but will not be dealt with in the present paper. I propose to discuss here the question as to whether the heated immune substance in the presence of

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* Some misunderstanding (Wright and Reid, 'Roy. Soc. Proc.,' 1906) has arisen in regard to my remarks ('Roy. Soc. Proc.,' vol. 76, p. 506) on the limitations of Wright and Douglas' technique. I would again point out that their method may fail to demonstrate sensitising substances where these are present in considerable amount, and may show no difference between two fluids, notwithstanding that one contains a large amount of sensitising substance, the other only a small amount, or none.

To illustrate this, I may quote some experiments which were carried out with the view of determining the limits of sensitising action in an immune staphylococcic serum. Dilutions of 1 in 100 of this immune serum, and 1 in 100 normal serum, when tested by the ordinary technique of Wright and Douglas, showed no difference in their action, which was practically *nil*. On the other hand, if one mixed a volume of these dilutions with an equal volume of a coccal emulsion, so that the ultimate dilution was 1 in 200, and placed the mixtures at 35°·5 C. for one hour, and, after re-emulsifying both, carried out the ordinary technique, a very marked difference could be determined. The results obtained with the same sera, at an interval of some months, by three different observers, who endeavoured to adhere to standard conditions as to emulsions, etc., were as follows :—

	Phagocytic index of	
	Normal serum.	Immune staphylococcic serum.
(1)	2·4	21·0
(2)	3·3	18·7
(3)	1·9	23·7

† After I had made a communication on the effect of dilution at the Pathological Society of Great Britain and Ireland, a paper by Simon, Lamar, and Bispham, which deals with the importance of this subject of dilution, came into my hands.

fresh serum produces a greater effect than results from the two substances acting independently; in other words, is the action similar to, or identical with, the action of a bacteriolytic amboceptor and complement? At least two methods may be employed in the study of this problem: one, the direct method, of estimating the activity of the bodies when acting separately and in combination; the other, indirect, by employing something which will throw out of action one of the constituents of a combination, leaving the other unaffected.

THE DIRECT METHOD.

Experiments with Staphylococci.

The ordinary technique was employed. In this experiment 1 volume of leucocytes, 1 volume of coccal emulsion and 1 volume of the serum were employed. The result was as follows:—

	Average cocci per leucocyte.
Normal serum of rabbit, fresh, 1 volume	11·9
Immune serum of rabbit, heated to 60° C. for 20 mins., 1 volume	7·1
1 part immune heated and 4 parts fresh normal serum, 1 volume	33·0

The calculated value of the mixture at the highest estimation does not exceed 19, whereas the experimental value is 33. Some other factor must therefore be at work: in this experiment probably a complementing of the immune heated serum by the fresh serum.

Another experiment of the same type may be recorded in which a slightly different technique was employed.

	Average cocci per leucocyte.
Normal rabbit serum, heated, 1 volume, + Immune staphylococcus rabbit serum, heated, 1 volume, + Normal salt solution, 1 volume, + Leucocytes, 1 volume, + Cocci, 1 volume.	3·0
Normal rabbit serum, heated, 1 volume, + Fresh normal rabbit serum, 1 volume, + Normal salt solution, 1 volume, + Leucocytes, 1 volume, + Cocci, 1 volume.	6·9
Normal rabbit serum, heated, 1 volume,* + Immune staphylococcus rabbit serum, heated, 1 volume, + Normal rabbit serum, fresh, 1 volume, + Leucocytes, 1 volume, + Cocci, 1 volume.	15·1

* This was added to control a factor in another experiment.

Here the calculated value does not exceed 10, whereas the experimental value was 15.

Experiments with the Dysentery Bacillus of Shiga.

In the case of such organisms as the Dysentery bacillus, Typhoid bacillus, and Cholera vibrio, a difficulty presents itself owing to the occurrence of bacteriolysis. If the full strength of certain fresh normal sera is employed, an accurate estimation of the phagocytosis by this method of investigation may be impossible on account of the fact that many of the bacilli disappear altogether or are visible only as shadow forms both inside and outside the leucocytes. These phenomena are still more pronounced when one mixes with such a fresh normal serum a heated immune serum of any potency. The intracorpuscular and extracorpuscular bacteriolysis in such an experiment may be extreme. By a process of dilution of both the normal and the immune serum it was found possible to overcome this difficulty. For example, after a number of orientating experiments with dysentery, cholera, etc., it was found that if a dilution of 1/100 of a particular sample of heated anti-dysenteric serum was employed with a dilution of 1/20 or 1/30 of fresh serum, marked phagocytosis occurred with slight intracellular but no obvious extracellular bacteriolysis. When 1 volume of these dilutions is mixed with 1 volume of leucocytes and 1 volume of bacterial emulsion, the ultimate dilutions reached are 1/60 or 1/90 of the fresh normal serum and 1/300 of the heated immune serum.

The procedure carried out in such an experiment is as follows. Tubes and preparations are made in the usual manner from the following combinations:—

	Average number of bacilli per leucocyte.
A. Heated anti-dysentery serum (dilution 1 : 100), 1 volume, + Normal salt solution, 1 volume, + Leucocytes, 1 volume, + Dysentery bacillus (Shiga), 1 volume.	4·3
B. Fresh normal serum (dilution 1 : 30), 1 volume + Normal salt solution, 1 volume, + Leucocytes, 1 volume, + Dysentery bacilli (Shiga), 1 volume.	5·6
C. Heated anti-dysentery serum (dilution 1 : 100), 1 volume, + Fresh normal serum (dilution 1 : 30), 1 volume, + Leucocytes, 1 volume, + Dysentery bacilli, 1 volume.	16·1

In Table III are shown the results of four such experiments with anti-dysentery serum and fresh normal sera of different ages, with the mean values obtained.

Table III.—Average Number of Bacilli Ingested per Leucocyte.

Experiment number.	A. Normal serum fresh. Dil. 1 : 30, ultimate dil. 1 : 90.	B. Anti-dysentery serum heated to 60° C. for 20 minutes. Dil. 1 : 100, ultimate dil. 1 : 300.	C. A mixed with B.
(1)	1·7	1·0	7·5
(2)	0·5	1·2	5·9
(3)	5·6	4·3	16·1
(4)	2·5	1·3	9·0
Total	10·3	7·8	38·5
Mean of averages	2·5	1·9	9·6

If the effect produced by the combined action of the normal fresh serum and the immune heated serum were merely additive, the calculated value should be 4·2, whereas the experimental value is 9·6. Taking into consideration the results obtained in the experiments upon the influence of dilution, we must assume that the mixture containing the higher serum concentrations should have given a lower count even than 4·2, whereas, by experiment, a count of 9·6 is obtained. Another factor, therefore, must be at work in the production of this result, in all probability the complementary of the immune serum by the fresh normal serum.

Experiments with the Typhoid Bacillus and the normal Sera of the Guinea-pig and of the Rabbit.

Certain normal sera have been very thoroughly investigated for the presence of amboceptor and complement in connection with bacteriolysis. It seemed of interest to examine some of these for sensitising and complementing action from the standpoint of phagocytosis.

If a like relation is proved to exist, it will be at least an indication that the substances which prepare the microbes on the one hand for extracellular bacteriolysis, and on the other hand for phagocytosis, are identical, and that free complement, while an essential factor only in the former process, yet plays in the latter also an important, if secondary, *rôle.*

The sera of the rabbit and guinea-pig, which have been investigated by Wechsberg (1902) and others for amboceptor and complement action in relation to the bacteriolysis of the typhoid bacillus, offer excellent material for a comparative study of the two processes.

The following experiment, which is a repetition and confirmation of an experiment of Wechsberg, shows how the matter stands for bacteriolysis in the case of the guinea-pig and of the rabbit.

Experiment.—A 24-hours' agar culture of a typhoid bacillus, recently isolated from the blood of a patient, was emulsified in bouillon and diluted. Into each of a series of small test-tubes there was introduced a quantity corresponding to 1/1000 of the whole bacterial mass. Serum was added in varying amount, and the volume in each tube made the same by the addition of normal salt solution. A small volume of broth was also added to each tube.

These tubes were then placed in the incubator for three hours, after which a fixed volume was taken from each and plated.

At the same time the various serum constituents were tested for sterility.

Table IV

Culture.	Fresh unheated guinea-pig's serum.	Normal rabbit's serum heated to 60° for 20 minutes.	Number of colonies on agar plate.
$\frac{1}{10000}$ of an agar culture 1 day old of *B. typhosus*	c.c.	c.c.	
	0·5	0	109,998
	0·25	0	uncountable
	0·1	0	uncountable
	0·5	1·0	4,365
	0·25	1·0	21,604
	0·1	1·0	uncountable
	0·0	1·0	uncountable

This experiment shows that inactivated rabbit's serum is capable of being reactivated by normal guinea-pig's serum, so that the combination of the two is capable of producing a marked bacteriolytic effect in quantities which, acting apart, have little or no such action. Comparative tests with fresh rabbit's serum and fresh guinea-pig's serum showed that the latter had much less bacteriolytic action than the former, and the above experiment proves that this difference is due to a deficiency of amboceptor.

Employing the opsonin technique, a number of tests was performed with the object of ascertaining how these two sera influenced the bacilli in regard to phagocytosis.

Experiment.		Phagocytic index.
1	Guinea-pig's serum, heated to 60° for 20 minutes	0·2
2	Rabbit's serum, heated to 60° for 20 minutes	0·4
3	Guinea-pig's serum, fresh	5·7
4	Rabbit's serum, fresh	10·5
5	Rabbit's serum (60° for 20 minutes) + guinea-pig's serum, fresh	15·6

All were brought to the same volume.

A repetition of Experiments 3, 4, and 5, with a thinner emulsion of the typhoid bacillus, gave the relation 0·9, 2·0, and 3·6.

These experiments show, in the case of the sera of the rabbit and of the guinea-pig in relation to the typhoid bacillus, a marked parallelism between the extra-corpuscular bacteriolytic function and the sensitising or opsonising function by means of which the organisms are prepared for phagocytosis, and that a complementary action takes place in both cases.

The Indirect Method.

These experiments were based on the observations made by Ehrlich, Morgenroth, Bordet, Pfeiffer, and others that the injection of the serum of one species into another gives rise to the elaboration in the blood of the latter of anti-bodies, which may act either on the amboceptor or on the complement. Anti-complement formation is a common phenomenon, whereas anti-amboceptor is of rare occurrence. The mechanism of the action of such sera has been the subject of much discussion which cannot be touched on here. In the present paper the term "anti-complement serum" will be used without prejudice, the possibility that such a serum may act on other bodies besides complement not being overlooked. In relation to the present subject, it seemed of interest and importance to ascertain the effect produced by an anti-complement serum in experiments of the type of these with which we are dealing. Whilst these experiments were in progress, Muir (1906) published results obtained by a similar method with which, in the main, the present series are in agreement.

Technique Employed.

The anti-complement serum was prepared by injecting a rabbit on several occasions with human or horse serum which was previously heated to 58° to 60° C. for half an hour. The anti-complement serum, after separation, was itself heated to 60° C. for 20 minutes. Varying proportions of this serum were then added to the homologous fresh serum, or to the mixture of which it was a constituent part. The tubes containing these were incubated for two hours at 35°·5 C. In this way a precipitin reaction was produced. The supernatant fluid removed from the precipitum was employed in the experiment. By doing a series of eight or 12 such tests, the most effective combinations of the serum were ascertained. In some of the experiments there was no such preliminary treatment of the fresh serum by the anti-serum, this being simply made one of the constituent parts of the mixture of leucocytes, bacilli, etc., and allowed to act during 15 or 20 minutes. The further carrying out of the experiments was as follows:—

(A) Normal rabbit serum, heated to 60° C. for 20 minutes, 10 volumes, +
Fresh normal horse serum, 5 volumes.
(At 35°·5 C. for two hours.)

(B) Anti-complement (horse) serum of rabbit, heated to 60° C. for 20 minutes, 10 volumes, +
Fresh normal horse serum, 5 volumes.
(At 35°·5 C. for two hours.)

(C) Normal rabbit serum, heated to 60° C. for 20 minutes, 10 volumes, +
Normal horse serum, heated to 60° C. for 20 minutes, 5 volumes.
(At 35°·5 C. for two hours.)

(D) Anti-complement (horse) serum of rabbit, heated to 60° C. for 20 minutes, 10 volumes, +
Normal horse serum, heated to 60° C. for 20 minutes, 5 volumes.
(At 35°·5 C. for two hours.)

The supernatant fluid from each of the above mixtures A, B, C, and D, was subsequently mixed with an equal volume of human leucocytes and staphylococcic emulsion and kept at 37° C. for 15 minutes.

When horse serum had been injected into the rabbit to obtain the anti-complement serum, it was found impossible to use horse leucocytes in the experiment, on account of the rapid agglutination of the red cells and entanglement of the leucocytes. In this case, therefore, human leucocytes were used, and by so doing the possible danger of a certain amount of specific leuco-toxic action was avoided.

The results of seven experiments are detailed in Table V (p. 410).

It is seen that a great loss in the sensitising power has resulted in Experiment 2, where the anti-complement serum was used. The higher count of Experiment 4, as compared with Experiment 3, is probably largely due to the fact that the heated anti-complement serum had a higher sensitising power than the normal rabbit serum employed, the ratio being about 2 : 1. This last observation applies to all the corresponding figures in the table, which summarises the results of seven such experiments, with a varying proportion of anti-complement and normal sera *versus* fresh horse serum. On comparing the second and fourth columns of that table, it will be seen that fresh normal serum, after having been acted on by the anti-complement serum, gives almost the same phagocytic average as is obtained by heated horse serum under the same conditions, *i.e.*, a loss has occurred comparable to that produced by heating to 60° C. for 20 minutes.

Table V.—Results of Experiments with Anti-complement (Horse) Serum obtained from Rabbit, together with Control Results.

Number of experiment.	Fresh normal serum of horse, +		Serum of horse heated (60° for 20 mins.), +	
	A. Normal rabbit's serum. Heated to 60° C. for 20 mins.	B. Anti-complement serum of rabbit. Heated to 60° C. for 20 mins.	C. Normal serum of rabbit. Heated to 60° C. for 20 mins.	D. Anti-complement serum of rabbit. Heated to 60° C. for 20 mins.
1	17·5	7·5	4·1	5·5
2	10·7	3·5	1·2	3·4
3	8·3	2·1	2·6	0·8
4	8·4	1·1	1·1	3·2
5	6·3	0·6	1·3	0·2
6	7·1	4·4	1·4	3·0
7	6·0	2·5	1·0	1·1
Total	64·3	21·7	12·7	17·2
Mean of experiments	9·1	3·1	1·8	2·4

The serum of the horse, after having been heated for 20 minutes to 60° C., still shows some sensitising power, even when tested by Wright and Douglas' method. Several comparative tests by my assistants and myself, where a large number of leucocytes had been counted, show that heated normal horse serum, compared with normal salt solution, gave average counts in the ratio of about 4 : 1.

Action of Anti-Complement Serum in Complemented Mixtures.

The following experiments with staphylococci may be reported in full as the type of such experiments. The usual opsonic technique was employed, the constituent parts of the mixture introduced into the tubes being as follows (see table, p. 411).

A comparison of 1 and 4 shows that, by the action of anti-complement serum, the phagocytic average has been reduced from 15·1 to 2·4.

Similar experiments with the dysentery immune serum of the horse complemented with normal human serum showed, as the result of the action of the anti-complement serum, a reduction in the phagocytic index in one experiment from 7·5 to 1·3, in another from 5·9 to 1·7. In this case human anti-complement serum and horse leucocytes were employed.

	Phagocytic index.
1. Normal rabbit's serum, heated to 60° C. for 20 minutes, 1 volume, + Immune rabbit's staphylococcus serum, heated to 60° C. for 20 minutes, 1 volume, + Normal human serum, fresh (dilution 1 : 10), 1 volume, + Leucocytes, 1 volume, + Cocci, 1 volume	15·1
2. Normal rabbit's serum, heated to 60° C. for 20 minutes, 1 volume, + Normal salt solution, 1 volume, + Normal human serum, fresh (dilution 1 : 10), 1 volume, + Leucocytes, 1 volume, + Cocci, 1 volume.	6·9
3. Normal rabbit's serum, heated to 60° C. for 20 minutes, 1 volume, + Immune rabbit's staphylococcus serum, 1 volume, + Normal salt solution, 1 volume, + Leucocytes, 1 volume, + Cocci, 1 volume.	3·0
4. Anti-complement rabbit's serum, heated to 60° C. for 20 minutes, 1 volume, + Immune rabbit's staphylococcus serum, heated to 60° C. for 20 minutes, 1 volume, + Normal human serum, fresh (dilution 1 : 10), 1 volume, + Leucocytes, 1 volume, + Cocci, 1 volume.	2·4
5. Anti-complement rabbit's serum, heated to 60° C. for 20 minutes, 1 volume, + Normal salt solution, 1 volume, + Normal human serum, fresh (dilution 1 : 10), 1 volume, + Leucocytes, 1 volume, + Cocci, 1 volume.	1·7
6. Anti-complement rabbit's serum, heated to 60° C. for 20 minutes, 1 volume, + Immune rabbit's staphylococcus serum, heated to 60° C. for 20 minutes, 1 volume, + Normal salt solution, 1 volume, + Leucocytes, 1 volume, + Cocci, 1 volume.	3·7

Summary.

1. Dilution of fresh unheated serum is not accompanied, so far as the higher concentrations are concerned, by a fall in the sensitising power for certain organisms (staphylococcus, tubercle bacillus). The diminution in this was found, as a rule, to begin at the quarter concentration.

2. So far as the present experiments go, the points corresponding to the 1/4th, 1/8th, 1/16th, and 1/32nd dilutions of normal human serum lie on a parabola whose equation is $y^2 = 4X$, *i.e.*, for these dilutions the phagocytosis is proportional to the square root of the serum concentration.

3. The phagocytic index obtained by mixing appropriate dilutions of a heated immune serum with a normal fresh serum is greater than results from the two substances acting separately.

4. In the case of certain normal sera (guinea-pig and rabbit) previously investigated for amboceptor and complement in relation to the extra-corpuscular bacteriolysis of the typhoid bacillus (Wechsberg), an exact parallelism can be demonstrated to exist between that function and opsonisation. The normal amboceptor can be complemented by fresh serum in regard to both functions.

5. An "anti-complement" serum, when mixed with a fresh normal serum alone or in a mixture containing heated immune serum, throws out of action the thermolabile substance, whereas it does not appear to influence the thermostable substance.

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ON PHAGOCYTOSIS

AND

BACTERICIDAL ACTION.

Being Introduction to a Discussion in the Section of Pathology at the Annual Meeting of the British Medical Association held at Exeter, July-August, 1907,

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METCHNIKOFF's theory of phagocytosis is one of the greatest conceptions of modern biology. Combated at the outset by many of the leading pathologists of the time it has steadily gained ground, and the main theses are now almost universally accepted. No description of infection, inflammation, or immunity which omitted to give a due consideration to phagocytosis would at the present time be considered as adequate.

If time permitted, it would be interesting to dwell on the ideas presaging this theory—the favourable nature of the inflammatory reaction (Boerhaave and John Hunter), the pus laudabile, the diapedesis of the leucocytes, especially in inflammation (Waller, Cohnheim), their capacity to take up small foreign bodies (Max Schultze), then the hypothesis that the leucocytes and certain other cells are the means of affording protection to the animal body, by attacking and destroying microbial invaders (Metchnikoff).

The nature of the processes involved in the mechanism of phagocytosis has been, and indeed is at the present time, the subject of much inquiry and controversy.

Metchnikoff and his pupils, while upholding the cell as the most important factor in the process of protection, could not long overlook the part played by the body fluids. The work of Nuttall and others on the bactericidal action of normal serum, and Pfeiffer's demonstration of the bacteriolysis of the cholera and typhoid bacilli by immune serums in the absence of cells, formed the basis for the humoral theory which attributed the chief rôle in immunity to the destructive action of the body fluids, serum, or plasma on the microbes, the phagocytes playing merely a secondary unimportant part, or perhaps no part at all. It was conceivable, indeed, that the body might be capable, apart from the phagocytes, of ridding itself of the completely bacteriolyzed bacilli by excretory processes. The evidence in favour of this bactericidal action of the serum was so conclusive and the fundamental experiments were so striking and easily confirmed, that for some time the phagocytic theory was overshadowed.

That an immune serum loses its bactericidal property by heating to 56° C., but is capable of being reactivated by fresh normal serum, peritoneal exudate, etc., is a fundamental observation which was first made by Metchnikoff and Bordet. The bactericidal action of the immune serum, then, is due to the combined activity of two substances—the one thermostable, the immune substance fixateur, substance sensibilisatrice, amboceptor, as it has been variously designated; the other thermolabile, the alexin or complement The same double nature has been demonstrated in the case of normal serum by Moxter, Wechsberg, and others.

According to Metchnikoff, the bactericidal action of the body fluids does not play an important part in the living animal under normal conditions. The blood plasma does not behave like the serum in this respect. The bactericidal substance has its origin in the leucocyte, and is set free by its death during the process of clotting, or, in the case of the living animal, the phagolysis is produced by injurious influences, such as an excessive dose of bacilli injected experimentally. This question is still an open one as to whether the blood plasma contains alexin or free complement. The belief that serum contains more bactericidal substances than plasma is largely based on the observation that a quantity of serum (for example, of the rabbit) which corresponds to only a small fraction of the plasma in the whole body, is capable of destroying a much greater number of germs than would constitute a lethal dose if injected directly into the blood stream. Important also was Levaditi's demonstration that when cholera vibrios are injected into the blood, if sufficient precautions are observed to avoid injury to the leucocytes, no conversion of the vibrios into the granular form takes place outside the phagocytes. Gengou's failure to obtain bacteriolysis with plasma prepared in paraffined tubes, in which case the leucocytes were undamaged, also gave a strong support to the view that extra-corpuscular bacteriolysis is a phenomenon secondary to phagolysis.

On the other hand, a number of workers, and quite recently Lambotte and Stiennon (1906), believe that, unlike Gengou and others, they have been able to obtain, without destruction of the leucocytes, a plasma which has bactericidal properties equal to those of the serum obtained from the same blood after coagulation.

It soon became apparent that the bactericidal action of the body fluids did not furnish a complete explanation of the resistance offered by normal and immune animals. As one example out of many in which the humoral theory did not fit the facts, the case of immunity against such organisms as the streptococcus, the pneumococcus, and the staphylococcus, may be instanced. No bacteriolytic activity could be demonstrated in the immune serums corresponding to these cocci, notwithstanding that the injection of such serums, even after the destruction of their thermolabile constituents, was capable of conferring immunity. A reaction therefore set in against the purely humoral view, resulting in the almost universal adoption of the phagocytic theory, which has grown by a process of natural evolution under Metchnikoff's guidance into a form which adequately explains many of the facts of immunity.

Two forms of activity in relation to phagocytosis have been attributed by Metchnikoff and his school to the thermostable immune substances : (1) a "stimulin" action, (2) a "fixative" action.

1. As was shown by Pfeffer, motile organisms of low organization are repelled by certain substances (negative chemiotaxis), attracted by others (positive chemiotaxis), and the same is found to be true of leucocytes. When a highly virulent microbe is injected into a normal animal the leucocytes do not show any great disposition to move towards or to attack the invader, which seems to repel or at least not to attract the phagocytes; in other words, the bacillus has a negative chemiotaxis towards the leucocytes. In an immunized animal, on the other hand, no sooner does the virulent organism find an entrance into the body than the leucocytes crowd to the site of infection, surround the invaders, and, if the immunity is sufficient, englobe and destroy the enemy. That this effect is due to something in the plasma or serum, and not to properties of the leucocyte acquired by education or otherwise, is shown by injecting some of the blood plasma or serum of the immune animal into a normal animal, in which case the leucocytes of the new animal, thus passively immunized, behave in the same manner as did those of the actively-immunized animal. Further proofs of this derived from other work will be dealt with later. Substances which thus appear to heighten the activity of the leucocytes by acting directly on them have been designated by Metchnikoff "Stimulins."

2. In his second view the serum is conceived as acting primarily, not on the leucocyte but rather on the microbe, on which a change is produced, so that it no longer repels but rather attracts the phagocyte; in other words, its negative is converted into a positive chemiotaxis.

These views of Metchnikoff's were largely based on work by himself and his pupils Bordet (1895 and 1897), Savtschenko and Melkich (1901), Levaditi (1901), and Savtschenko (1901), Tarassevitch (1902).

THE INFLUENCE OF SERUM ON PHAGOCYTOSIS.

It would be impossible to detail or even to refer to the chief of the earlier contributions bearing on this subject of the influence of the serum on phagocytosis. I have already in a former paper given a brief *résumé* of some of the more important. For the sake of continuity of argument I must refer in the briefest terms to a few.

Denys and Leclef (1895) and Denys (1897) showed in the case of the streptococcus, and Mennes (1897) in the case of the pneumococcus, that the increased bactericidal action of the immune blood was due to phagocytosis, which did not depend on a change in the leucocytes, but was due to a property of the serum, for the leucocytes of the immune animal when put into normal serum were no more active than normal leucocytes. Markl (1903) submitted plague bacilli to the action of heated immune serum, which was then removed by washing and centrifugalizing. The bacilli having been treated in this manner when injected into an animal were found to behave in the animal body exactly like bacilli which had been injected along with immune serum. He therefore concluded that the immune serum had a direct influence on the bacilli When bacilli of low virulence are injected, extracellular bacteriolysis plays an important part, whereas where highly virulent bacilli are employed in the experiment phagocytosis is the only agency at work in their destruction. Many of Markl's experiments were repeated and confirmed by the writer.

Fresh interest has been given to these questions, especially in our own country by the work of Wright and Douglas, who by a modification of the beautiful method devised by Leishman have made a number of observations emphasizing the importance of the serum in the mechanism of phagocytosis, and showing for the first time in an unequivocal manner that normal serum, as compared with indifferent fluids—such as normal salt solution—has a powerful action in preparing bacteria for phagocytosis, even in cases where no bacteriolytic or bactericidal action is apparent The substance in serum which produced this action was found by them to be thermolabile, and was designated by them "opsonin." In the case of furunculosis, tuberculosis, etc., Wright and Douglas showed that the injection of vaccines made from the appropriate micro-organism is followed by an increase in the opsonic power of the blood. The fascinating method and the interest of the subject have attracted many workers. The fundamental observation that normal serum as compared with other indifferent substances opsonizes the microbes has been repeated and confirmed by many. Much critical investigation, however, will be necessary before the establishment of the claims made as to the value of the method for clinical use. Bulloch and Atkin (1905), Hektoen and Ruediger (1905), and others confirmed and extended these results.

About the same time Neufeld and Rimpau (1904) described in antistreptococcic and in antipneumococcic serum substances which they named "bacteriotropic" substances, or "bacteriotropins," which are thermostable, and which promote phagocytosis, not by stimulating the leucocytes but by acting directly on the microbes.

Leishman (1905) attributed a stimulin action to thermostable substances in the serum of enteric and Malta fever cases, and in the immune serums obtained by treating animals with the causal organisms of these diseases.

Dean (1905) found independently that in certain immune serums, for example, in antistaphylococcus, in antidysentery, and in antityphoid serums, there are present thermostable substances which by their action prepare the homologous organism for phagocytosis—that is, act like opsonins. On the basis of Ehrlich's theory, a small quantity of this substance ought to be present in normal serum, and on this being put to the test such a substance was demonstrable.

This result taken along with certain other experimental data seemed to indicate that the thermostable substances of normal and of immune serums were probably identical. It was further shown that when submitted to a temperature of 60° C. both immune and normal serum suffered a sudden diminution in opsonic power. This change occurs in the first few minutes of heating, after which further loss takes place slowly. It was not overlooked that these results suggested that the sensitizing action of the serum might be due to the combined action of two substances, the one the essential substance, thermostable, the other thermolabile—the one of the nature of the substance sensibilisatrice or amboceptor, the other of the nature of an alexin or complement.

Wright and Reid (1906) found that in the serum of patients suffering from tuberculosis there may be present a considerable amount of thermostable opsonin. Macdonald (1906) found that immune antipneumococcic serum had varying degrees of thermostability, and Rosenow (1906) holds that the pneumococco-opsonin is quite resistant to heat.

Löhlein (1905) has endeavoured to show that phagocytosis can take place in the absence of serum when the leucocytes and the microbes are suspended in salt solution only, especially when longer periods of action—say, one or two hours—are allowed. I have done a number of experiments in this direction, and have found that, as a rule, the phagocytosis is trivial with salt solution as compared with serum, a result which, I believe, is in accordance with the experience of most observers.

A number of workers have investigated phagocytosis by employing an immune serum obtained from an animal which has been previously injected with the erythrocytes of another species—Savtschenko (1902), Tarassevitch (1902), Levaditi (1903), Gruber (1903). Such a serum is, as a rule, agglutinating and haemolytic for the homologous red blood cells, but Neufeld and Töpfer (1905), Barrett (1905), and Hektoen (1906) hold that the thermostable haemotropic substances or haemopsonins are distinct from agglutinins and amboceptors, because a serum may be lytic and agglutinating for the homologous erythrocytes, but not haemotropic or opsonizing. Keith (1906) admits an amboceptor-like action, but postulates a special thermostable opsoniphoric group to account for the phagocytosis which takes place with the heated serum in the absence of complement.

The chief difficulty in accepting the differentiation of haemotropin from haemolysin is, as it appears to me, that the same substance acting in different concentrations may be capable of producing an effect sufficient in the one case for phagocytosis, in the other for haemolysis. Though such work may give valuable indications in regard to the phagocytosis and bacteriolysis of microbes the absence of the factor of virulence, of the importance of which I shall speak later, makes it unjustifiable to completely transfer all the deductions obtained from the one field to the other.

Muir and Martin (1906 and 1907) have undertaken a very complete study of the combining properties of the thermolabile opsonins of normal serum and of the thermostable opsonins of immune serums.

In the case of the thermolabile substances of normal serum they have shown that various substances or combinations of substances which absorb complements also absorb opsonins. Red-blood corpuscles, bacilli, and serum when combined with their corresponding antibodies — namely, haemolytic and bacteriolytic amboceptors and precipitins —in absorbing complement are found at the same time to have absorbed opsonin. The normal thermolabile opsonins, therefore, belong to the group of complements, and, like these, have no specific properties in their combining affinities. They found a great reduction in the staphylococcic opsonin on treating normal rabbit's serum with the tubercle bacillus, a result in agreement with the work of Simon, Lamar, and Bispham (1906), and Yorke and Smith (1906), and at variance with that of Bulloch and Western (1906), a divergence probably dependent on a more complete exhaustion of the serum by the use of a larger amount of tubercle bacillus. On the other hand, an immune staphylococcus serum treated with a like amount of tubercle bacillus showed no reduction in its opsonizing action for staphylococci. Similarly other powerful complement absorbers, such as sensitized erythrocytes or bacteria, have no effect on the thermostable immune substance.

The thermostable immune opsonins possess the comparatively specific characters of antibodies generally. It is left an open question whether it is an agglutinin or an immune substance, though Muir and Martin believe that certain facts point in favour of the former. They conclude that the thermolabile opsonin of normal serum and the thermostable opsonin of immune serum are two distinct classes of bodies.

Neufeld and Hüne (1907) have extended the investigation commenced by Neufeld and Rimpau and Neufeld and Töpfer. From the results obtained with normal and immune serum in the case of the staphylococcus, the typhoid bacillus, and certain members of the paratyphoid bacillary group, they conclude that the bacteriotropins and the bacterio-opsonins are distinct bodies, that the bacteriotropic action of immune serum has nothing to do with the opsonic action, which is dependent on the presence of complement and small quantities of normal amboceptor.

In admitting that amboceptor and complement are capable of acting on the bacilli, so that these, though not killed, have been so far damaged that they can be taken up by the leucocytes, Neufeld and Hüne appear to me to have given away their case for a separate bacteriotropic substance. For if amboceptor and complement are capable of producing the effect in the one case, why not in the other?

Dean (1907), in a second paper, dealt with the influence of dilution of the serum, an important question into which time does not permit me to go at any length here. I should like, however, to indicate that the adoption of a method based on dilution and on the estimation of the point in a series of dilutions at which a serum ceases to give evidence of a distinct opsonizing action, as compared with a normal serum, would lead to a great improvement in the accuracy and consequent value of the results. Such a method is very laborious, but I am hopeful that it may be possible to simplify the necessary processes. It was found, as had been anticipated, that the phagocytic index obtained by mixing appropriate dilutions of a heated immune serum with a fresh normal serum is greater than results from the two substances acting separately.

In the case of certain normal serums — guinea-pig and rabbit—previously investigated for amboceptor and complement by Wechsberg and others in relation to the extra-corpuscular bacteriolysis of the typhoid bacillus, a parallelism can be demonstrated to exist between that function and opsonization. The normal amboceptor can be complemented by fresh serum in regard to both functions. An anticomplement serum, when mixed with a fresh normal serum alone, or in a mixture containing heated immune serum, throws out of action the thermolabile substance, whereas it does not appear to influence the thermostable substance.

That there is amboceptor in normal serum, and that this is concerned in the process of opsonization, has further been confirmed by certain experiments of Ledingham's (1907), who found that heated normal serum which has been in contact with tubercle bacilli, after the removal of these by centrifugalizing, has acquired the property of greatly inhibiting the opsonic action of fresh normal serum not only towards the tubercle bacilli, but also towards the *Staphylococcus pyogenes aureus.*

This phenomenon is due, he believes, to there being given off from the bacilli into the heated normal serum receptors, which combine with the normal amboceptors. This receptor-amboceptor combination, acting in the same way as the similar combinations referred to under Muir and Martin's work, withdraws the complement from the fresh serum and thus inhibits opsonization.

Levaditi (1907), in a series of communications, along with various collaborators, published about the same time as Dean's, arrived at practically the same conclusions. I shall not deal with them here, but shall merely refer briefly to one or two of his experiments.

It has been shown that the aqueous humour of the rabbit does not contain complement. Levaditi, on testing it, found that in it there was practically no opsonin. The fluid which is thrown out into the anterior chamber in two or three hours after the first puncture may contain appreciable quantities of complement or none. In two cases where this aqueous fluid of second formation was tested, the one, rich in complement, was also rich in opsonin, the other, poor in complement, was also poor in opsonin. A like parallelism was found in the case of oedematous fluid.

PHAGOCYTIC ACTIVITY OF THE POLYMORPHONUCLEAR LEUCOCYTE.

As already indicated, Denys and Leclef demonstrated clearly both *in vitro* and *in vivo* that in the case of animals immunized against the streptococcus the immunity was due to increased phagocytosis which had its origin from a change, not in the leucocytes, but in the serum. This view that the leucocyte is in a sense an indifferent factor has been widely confirmed, notably recently by Wright and Douglas and many of those who have followed their work, and is in the main probably true. It is proper, however, to refer to a few observations which show that under certain conditions the leucocyte may be a variable factor.

Ledingham (1906) found in certain cases of myelogenous leukaemia that the serum may have quite a normal opsonic content as tested with normal leucocytes, but a markedly subnormal one if tested with the patient's own leucocytes. On the other hand, in a case of chronic lymphatic leukaemia the polymorphonuclear leucocytes were much more actively phagocytic than normal leucocytes. He also found, in collaboration with Bulloch (1906), that during the period of hyperleucocytosis produced by an injection of cinnamate of soda, a hyperleucocytosis accompanied by rise of complement but not of opsonin. These cells had much less phagocytic activity than normal leucocytes.

Rosenow (1906) holds that the pneumonic leucocytes are more actively phagocytic than normal leucocytes, a property which seems independent of the action of the serum. They are more resistant to heat, and when heated to the thermal death point and resuspended in serum they are more actively chemiotactic for pneumococci, which tend to collect round the dead leucocyte just as if by a form of agglutination of the microbes towards the leucocyte.

THE VIRULENCE OF THE MICROBE.

The virulence of the microbe is a factor of the highest importance in relation to phagocytosis and bactericidal action.

Pasteur, Leclef, and many others have shown that virulent microbes are more resistant than avirulent to the action of normal serum. Bordet, Pfeiffer, and Kolle pointed out that more immune serum is necessary to bacteriolyze a virulent than an avirulent typhoid or cholera bacillus. According to Pfeiffer and Friedberger (1905) this may be due to virulent bacilli possessing either more receptors or receptors which have a stronger combining affinity. The influence of virulence on phagocytosis was very thoroughly studied by Marchand (1898) in the case of the streptococcus.

Marchand found both in test tube experiments and in the living body that the leucocytes readily englobed the avirulent, but refused to touch the virulent cocci. By various ingenious experiments he arrived at the conclusion that this was not due to a secretion from the microbes, but depended on some physical condition associated with virulence. The organisms might be boiled, put in acid, alkali, or alcohol, and still comport themselves as virulent microbes. He therefore declines to accept the view of a negative chemiotaxis. The indifference of the leucocyte towards the virulent organism is not due to any paralysis of its functions.

Notwithstanding Marchand's opinion it is possible that the toxic particles are capable of surviving this treatment and are active towards the leucocyte in a chemical sense.

More recently Hektoen (1906) and Rosenow (1906) have investigated and emphasized the importance of the relation of virulence to phagocytosis in the case of the streptococci and the pneumococci.

In this connexion an interesting observation was made by Löwenstein (1906), who found in a case of genito-urinary tuberculosis that the leucocytes of the pus in the urine refused to phagocytose the tubercle bacillus also present in the urine, but readily under parallel conditions took up other strains of tubercle bacilli. He believes that this state of affairs was not due to the leucocytes or to the bacilli alone, but to a definite relation which the one had to the other.

Bail and Rubritius (1907) found that in the case of the typhoid bacillus there is a marked difference between bacilli derived directly from the animal body and those which have undergone artificial cultivation. There are slight morphological changes perhaps analogous to the capsule formation observed in the streptococci by Bordet (1897), in the anthrax bacillus by Sobernheim (1899) and others, but the most striking difference lies in the fact that the "animal" bacilli, unlike the culture bacilli, are refractory to the bacteriolytic action of the serum. Into the "aggressive" theory of Bail I cannot enter here, although it may have some relation to the question of opsonins. Indeed, Pettersson (1905) suggests that the opsonins may be identical with the antiaggressins. The question may well be left till the position of the aggressins as actualities is more securely established, for the investigations of Wassermann and Citron (1905), Doerr (1905), Kraus (1906) and others seem to show that they are probably identical with endotoxins or with free receptors or free toxic particles which may be obtained from bacilli by the action of serum and salt solution, or by autolytic processes. That such free receptors may inhibit bacteriolytic action we have ample proof from the work of the authors quoted.

It may be of interest to refer briefly here to certain experiments which I have carried out recently on the question of the virulence of the typhoid bacillus to its phagocytosis, a continuation of the work to which I have referred. These experiments will be published in full elsewhere.

In comparing the phagocytic index obtained with a particular serum in the fresh state and after heating, it was found that in certain cases the index was higher with the heated than with the unheated serum, as had been shown by Leishman—for example, in testing a normal human serum the ratio of the indices might be so:

$$\frac{\text{Phagocytic index of heated serum}}{\text{Phagocytic index of unheated serum}} = \frac{3}{1}$$

This I will designate the "reversed ratio." In other cases the ratio was always in the opposite direction, the heated serum giving a very low count, which is what one finds generally, and is therefore a "normal ratio," for example:

$$\frac{\text{Phagocytic index of serum heated}}{\text{Phagocytic index of serum unheated}} = \frac{1}{5}$$

It soon emerged that the former type of ratio occurred only with old laboratory cultures, the latter with several strains recently isolated from the blood of patients.

Presumably, then, it was a question of virulence, and on testing a number of strains of both types on guinea-pigs a definite correspondence could be determined. It was only, therefore, the avirulent strains that gave a higher index with heated normal serum. Indeed, the results have been so uniform that I believe by ascertaining the ratio one can state fairly definitely whether one is dealing with a virulent or an avirulent race. It was also shown by experiment that these recently isolated virulent strains were extremely refractory to the bactericidal action of serum, as was first noted by Pfeiffer and Kolle. In the case of some strains, even though they had been cultivated on artificial media for several generations, it appeared that the resistance was similar in degree to that observed by Bail and Rubritius in the case of their "animal" bacilli. It is therefore probably unnecessary to assume, as they do, that to obtain such bacilli they must be taken direct from the animal body.

On the other hand, the old laboratory avirulent strains were susceptible to the bactericidal action of serum. Now this bactericidal (bacteriolytic) action of the serum might act in several ways to produce the effect of the reversed ratio.

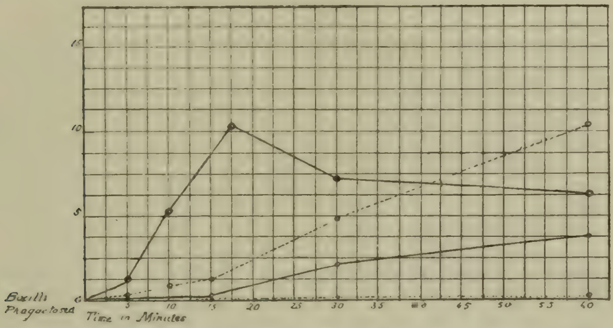
(1) It might reduce the number of bacilli outside the leucocyte, so that in the case of the unheated serum there would be fewer bacilli within its field of action for the leucocyte to englobe; or (2) it might bacteriolyse the bacilli outside the leucocyte, bringing them into a finely granular or invisible state, in which condition they might be taken up by the leucocyte to the exclusion of more or less intact and countable organisms. (3) In this finely-divided state the bacilli might have a certain toxic action on the leucocyte, thus hindering phagocytosis; or (4) it might, by aiding the process of internal bacteriolysis or digestion, reduce the number inside the corpuscles. That the last is an important factor was proved by several series of experiments, allowing different times for the phagocytosis to occur.

I will quote the ratios obtained in one series, done at 37° C.

	20 Min.		15 Min.		10 Min.		5 Min.	
	Vir.	Avir.	Vir.	Avir.	Vir.	Avir.	Vir.	Avir.
Heated serum	1	4.7	1	1.8	1	1	1	1
Unheated serum	20	1.0	10	1.0	8	1	6	4

In shortening the time the reversed ratio is changed to a normal ratio in the case of the avirulent organism, because there is no time within the five minutes for intracorpuscular bacteriolysis to occur.

The result of an experiment carried out at a rather lower temperature, so that the processes might occur more slowly, and thus be more easily followed, is shown on the Chart. It will be seen that in the case of the avirulent organism, where unheated serum is used, there is a sudden rise followed by a slow fall; whereas, in the case of the heated serum, there is a steady rise. In the case of the virulent, the phagocytosis is much less marked, but there is a steady rise in the case of the fresh unheated serum, whereas with the heated serum the phagocytosis remains very low. We may deduce, then, that the cause of the phenomenon of reversed ratio is an *increase* in the intracorpuscular bacteriolysis due to the action of the serum. The microscopical appearances observed are in agreement with this view.



Upper uniform line represents phagocytosis of avirulent typhoid bacilli in the presence of unheated serum.

Upper dotted line represents phagocytosis of avirulent typhoid bacilli in the presence of heated serum.

Lower uniform line represents phacocytosis of virulent typhoid bacilli in the presence of unheated serum.

Lower dotted line represents phagocytosis of virulent typhoid bacilli in the presence of heated serum.

The explanation thus afforded enables one to discount certain results at first obtained, which appeared to show that the bacteriolytic and opsonizing function of a serum did not correspond in the case of the typhoid bacillus. So far as my experiments at present go, they show a very good general correspondence between the two functions. A serum which gives a good bacteriolysis gives a good opsonization, and the converse; and that a bacillus which is difficult to bacteriolyze is also difficult to opsonize, and its converse, are all found to hold good. Too exact a correspondence must not be demanded, for the limitation of the methods must be borne in mind. The scales of measurement in the two are not identical. There is a difference in the relative delicacy of the methods in recording the degree of change produced on the microbe.

In testing a serum, therefore, if one were to take a highly virulent typhoid bacillus, one might arrive at the conclusion that the serum contained no typhoid bacteriolysins, but that it did contain typhoid opsonins, whereas by using a bacillus of lower virulence the presence of both lysins and opsonins could be demonstrated. That the bacteriolytic and bacteriotropic functions run parallel can be best shown by employing for the tests bacilli of both types.

The stronger a serum is in relation to the bacillus, the greater is the tendency for it to produce the reversed ratio. By selecting, therefore, a bacillus of such virulence that it gives a normal ratio with normal serum, one can form a rough estimate of the strength of stronger serums by observing the tendency to approach or reach the reverse ratio. Such a procedure may prove a useful adjunct to other methods of ascertaining the bactericidal action of typhoid serum.

In the discussion of such a question as this nothing could be more out of place than dogmatism. At the same time, the bulk of the evidence, as sifted from the literature and derived from my own experiments, drives me to the conclusion that the opsonizing action is due to two substances—the one thermostable, the substance sensibilisatrice or amboceptor; the other thermolabile, the alexin or complement. The thermostable substance is the essential one, and it may act alone, but its activity is increased by the presence of free complement. The amboceptor is present in relatively small quantities in normal serum, hence the apparent thermolability of the opsonin in normal serum, whereas in the case of an immune serum the amboceptor is present in a larger amount, and perhaps with heightened specific properties, and plays a predominant part, and though heating results in a loss of activity this is only partial. In both cases the loss is due to destruction of complement.

The reason why one type of organism, such as the typhoid bacillus, is bacteriolyzed by the action of these substances, whereas another, such as the staphylococcus, though prepared for phagocytosis, shows no bacteriolytic effect, probably depends not on any fundamental difference in the substances in the serum which produce the effects, nor in their method of action, but from a difference in the physical or chemical nature of the microbe itself.

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THE SPECIFIC PROPHYLAXIS AND TREATMENT OF BACTERIAL DYSENTERY.

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THE object of the following brief article on the subject of dysentery is to endeavour to arouse some practical interest in the advances which have been made with regard to the specific prophylaxis and treatment of the disease. A considerable amount of scientific energy has been expended on the subject during the past few years, but the results obtained would appear to have aroused little attention in this country. Only very meagre reports of clinical tests as to the value of specific remedies have so far appeared.

Many opportunities must occur to members of the Naval, Military, Indian and Colonial Medical Services to investigate the causation of outbreaks of dysentery, and in suitable cases to test the value of the methods of treatment which have been suggested as the result of recent experimental investigation. In view of what has been already published by many workers on the subject, such tests appear highly desirable, and these could be most satisfactorily made during small epidemics, and not in time of war, when stress of work must make it extremely difficult to arrive at accurate conclusions as to the value of a particular remedy or method of treatment.

It is almost unnecessary to refer here to the importance of the group of diseases included under the designation "dysentery." "*It is one of the four great epidemic diseases of the world.*"—Osler. In civil life in various regions it constitutes an ever-present danger to life and hindrance to activity, and even where under ordinary circumstances it is comparatively rare, famine or defective food or water supply may cause it to assume a serious epidemic character.

Its gravity in war has been constantly recorded, from the time of the pestilence during the Peloponnesian war, down to most recent times. A striking example of the terrible effects produced as a war pestilence, occurred during the War of Secession of the United States. Woodward states that among the Federal troops the number of deaths due to dysentery and diarrhœa was 37,794, or nearly 30 per cent. of the whole mortality, and among the prisoners at Andersonville the deaths from this disease amounted to 58·7 per cent. of the deaths from all causes. In many other recent wars the havoc worked by the disease has been hardly less appalling. In the

Franco-Prussian War there were 38,652 cases (= 49 per 1,000), and of these 2,380 died (=6·2 per cent. of those affected). Among the officers there were 223 cases with 25 deaths. The death-rate alone, however high, is an inadequate measure of the importance of the disease to armies in the field, for the number invalided is usually very high in proportion to the death-rate.

In this connection it is necessary to refer briefly to some of the most important facts which have been established with regard to the pathogenesis of dysentery. As early as 1873 Loesch had demonstrated the presence of amœbæ in the ulcerated gut in dysentery, an observation confirmed in Egypt by Koch and extended by Kartulis, who came to the conclusion that amœbæ were the sole cause of tropical dysentery. Notwithstanding the fact that amœbæ can occasionally be demonstrated, especially in certain regions, in the dejecta of normal individuals, the important *rôle* played in the pathogenesis of a certain type of dysentery by amœbæ, probably of a particular species—*Amœba histolytica*—is now pretty generally admitted. On the other hand, in many epidemics of dysentery, especially in temperate regions, the most careful investigation failed to demonstrate the presence of amœbæ.

From such observations it became probable that dysentery was not due to a single cause, but that other agents besides the amœbæ may be concerned in the production of the dysenteric condition. Numerous communications were made, suggesting a bacillary origin of many cases of dysentery, but nothing of a convincing nature appeared until the work of Shiga in 1898, when he succeeded in isolating a bacillus, which he regarded as the cause of the disease, from a number of cases during an epidemic in Japan, which from June to December of 1897 claimed 22,300 victims out of 89,000 cases (= 24 per cent.).

The same organism was found in Germany independently by Kruse, and wherever dysentery epidemics were investigated this bacillus, or bacilli closely related to it, was demonstrated, and its causal relationship to certain outbreaks of the disease is now generally admitted. It appears to be the cause of certain outbreaks of "Asylums' Dysentery" in this country, as was first shown by Eyre. In 1900, Flexner and Strong isolated from dysenteric stools in the Philippine Islands bacilli which they thought at the time to be identical with the Shiga bacillus, but which later were found to differ in several characteristics, such as in possessing the power of producing indol and of fermenting mannite. That this bacillus has a causal relation to certain outbreaks seems probable, but whether it must be regarded as a separate species or a mere variety of the Shiga type, is still an open question.

Regarding the subject from the point of view of what is known as to its causation, we must clearly separate amœbic dysentery from bacillary dysentery, and under bacillary dysentery it is well provisionally to differentiate at least the two types, *e.g.*, Shiga-Kruse type and the Flexner-Strong type. It is quite conceivable, and is indeed probable, that several organisms besides those referred to may be capable of producing dysentery: but the frequency with which these bacilli occur, and their wide distribution, made it desirable that their presence should be excluded before admitting a pathogenic *rôle* to another organism.

In regard to the amœbic type we have, so far, no knowledge of a specific prophylaxis or treatment, but in regard to the bacillary type we have a considerable amount of definite information with which I now propose to deal.

Prophylactic Inoculations of Bacillary Vaccines.—Shiga carried out on himself an experiment in this direction on the lines first suggested by Pfeiffer and Kolle in the case of typhoid and cholera. He injected subcutaneously a suspension (about half an agar tube) of bacilli killed by heating to 60° C. for twenty minutes. The local and general reactions following the injection were much more prolonged than in the case of typhoid and cholera inoculations, and Shiga concluded that this method was not likely to be suitable for general use. He therefore carried out some experiments on animals with the object of testing the value of a combined method in which crushed bacillary bodies were injected along with an immune serum. He obtained satisfactory results in the guinea-pig, and found that in that animal the immunity lasted for three weeks.

In man he injected one-half öse of a killed agar culture along with 0·5 cc. of an immune serum of high value, followed in three to four days by the injection of twice the amount of culture without serum. The reaction by this method was much diminished, and protective substances could be demonstrated twenty to thirty days after the injections. About 10,000 individuals in the area affected by the disease were inoculated by this method, but, unfortunately, the records were unsuitable for statistical investigation. As far as the facts permitted a deduction to be made, it appeared there was no marked effect produced upon the incidence of the disease, but that the mortality fell from 30 to 40 per cent. to almost nil. From this experience Shiga concluded that more reliance was to be placed upon a serum prophylaxis and treatment than on the employment of bacillary vaccines. It is possible, however, that in chronic cases a combined serum-vaccine treatment on these lines might give good results.

Treatment by Specific Serum.—Shiga was the first to prepare an anti-dysentery serum by injecting horses with the bacillus which he had isolated. Using 10-20 cc. doses of this serum in 300 severe cases of dysentery the death-rate was about 7 per cent., whereas in 200 cases of the same outbreak which were treated by ordinary methods the death-rate was 22 per cent. The serum appeared to have a distinct effect in reducing the number of evacuations and in shortening the duration of the disease to about one-half.

Kruse obtained similar results in Germany in a small number of about 100 cases. The mortality fell from about 11 to 5 per cent.

Both authors attribute the action of the serum entirely to a bactericidal effect, and the experimental data on animals were not of a very convincing nature. Shiga pointed out that this was the first serum which, when injected subcutaneously, had been demonstrated to possess a curative action on a disease of the alimentary canal, and contrasted the results with those obtained by similar means in typhoid or cholera. He laid great stress on the fact that the anti-dysentery serum found a suitable complement in human serum. Neither Shiga nor Kruse made any statement as to the possibility that the bacillus produced a toxin capable of stimulating the animal body to the formation of an antitoxin. Indeed, Kruse states definitely that in this case there was no question of the serum being antitoxic, "since neither do the bacilli secrete an especially powerful toxin nor does the course of the disease, with rare exceptions, present the picture of an intoxication."

A most important addition to the knowledge and understanding of the whole subject of bacillary dysentery and a stimulus to the hopefulness of arriving at a successful serum-treatment of the disease were given by the work of Todd in the Lister Institute, and of Rosenthal in Moscow. These workers independently showed that it was possible to obtain from cultures of the Shiga-Kruse bacillus a soluble toxin, and that the serum obtained by immunising animals with this toxin possessed definite anti-toxic properties. Their results were confirmed by Kraus, and Kraus and Doerr in Vienna.

To refer somewhat more in detail to these results, it was found that the filtrates from cultures about one month old which had been grown upon a particular alkaline broth, when tested on rabbits showed a very definite toxic action after a latent period of from one to three days. Severe diarrhœa sets in with paralysis, at first of the hind and then of the fore limbs, and ultimately of the muscles of the trunk and neck. The animal loses weight and dies in from one to five days. The chief naked-eye lesions are found in the large gut, which contains a slimy mucous fluid, with congestion, hæmorrhages and even erosions of the mucous membrane. The lungs are congested and sometimes small hæmorrhages are present. The rabbit and horse are very susceptible to the action of the toxin; the guinea-pig, rat, mouse and monkey much less so. The results with the Shiga-Kruse bacillus were in sharp contrast to those obtained with the Flexner bacillus, in which case under parallel conditions the filtrates possess a very feeble or no toxic action, *e.g.*, whereas 0·1 cc. of Shiga-Kruse filtrate was a lethal dose for a half-grown rabbit, 5 cc. of Flexner filtrate had little or no effect upon an animal of the same weight.

It is impossible here to enter into the subject of the immunisation of horses, &c., suffice it to say that after a fairly prolonged immunisation, either with the toxins from old alkaline broth cultures, or with the bodies of the young bacilli, the serum obtained from horses possesses antitoxic properties of a high order. The fact that bacilli from young cultures on agar were able to stimulate the animal body to the formation of antitoxin, and certain other considerations, led Todd to the conclusion that the toxin was of intracellular origin. In fact, this work on the dysentery toxin and antitoxin is one of the chief grounds which make it extremely difficult to maintain some of the older views as to the sharp line of demarcation between the toxins proper and the endotoxins.

The Properties of Dysentery Serum.—As already indicated, from the outset of the work on this subject it was recognised that the serum possessed bacteriolytic and agglutinating properties towards the homologous bacillus. A serum prepared with the Kruse bacillus appears to have not only specific, but also group bacteriolysins and agglutinins, which act on the Flexner type of bacillus, though to a much less extent than on the Kruse-Strong type employed in the immunisation.[1] As was shown by the writer, the opsonising action of the serum depends on the bacteriolytic property. From the point of view of therapeutics the antitoxic property is probably the most important quality of dysentery serum. To illustrate the high antitoxic value which the dysentery serum may reach, it may be stated that Todd found that when the toxin and antitoxin were mixed some time before injection, $\frac{1}{1000}$ cc. of serum completely protected a rabbit against twenty certain lethal doses of toxin. The serum is also capable of protecting animals when injected apart from the toxin, and even when injected some time after the toxin, and more recently Kraus and Doerr have found that in certain cases, it is possible by large doses of serum to save the life of the rabbits after the onset of a paralysis which proves fatal to the control animals. From the experimental standpoint, therefore, this serum must be classed along with diphtheria, tetanus and botulismus antitoxins, no serum, with the exception of these, possessing toxin-neutralising properties of the same quantitative order.

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[1] In the preparation of the Lister Institute serum, both types of bacilli are now employed.

The serum has now been used in a considerable number of cases in the human subject in addition to those to which reference has already been made. Rosenthal treated 157 cases with serum. During this epidemic the death-rate of cases treated by ordinary methods was 10-11 per cent., whereas the death-rate with serum treatment was only 4·5 per cent. The quantity of serum given varied from 20-40-60 cc., according to the severity of the case. This is the dosage now generally recommended.

In the Russo-Japanese war Korentchewsky employed Rosenthal's serum in 70 cases with good results.

Barijkin treated 59 cases in Manchuria with only 1 death.

Vaillard and Dopter treated 96 cases, all of a severe type, with 1 death. They classify their cases as follows :—

	Cases	Deaths
Cases of moderate severity ..	50 ..	0
Severe	18 ..	0
Grave	24 ..	0
Extremely grave	4 ..	1

Several other writers, Birt, Lüdke and others, have treated small groups of cases with favourable results. Kraus and Doerr obtained permission from the Austrian War Ministry to employ the serum in the Austrian Army. They give a brief history of eight cases treated with the serum and refer to other groups of cases in various garrisons. It was impossible to obtain, in many of the outbreaks, reports suitable for statistical analysis, but in all cases the results obtained with the serum produced a favourable impression upon the clinicians. The pain and tenesmus were controlled as by a narcotic, the stools in many cases rapidly assumed a natural form, the general conditions were ameliorated, and the period of illness was much shortened.

To sum up, there appears to be a strong case for the use of anti-dysentery serum as a prophylactic and therapeutic agent. The employment of the serum without reference to the nature of the disease is to be deprecated, as it can only lead to disappointing and misleading results. In every case, or at least in every outbreak, it is essential to ascertain the causal agent at work, whether the disease is of an amœbic or bacillary type, and also in the latter case to determine, if possible, to which group the bacillus belongs.

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N° 18

A TYPHOID CARRIER OF TWENTY-NINE YEARS' STANDING.

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THE following case seems worthy of record from its almost unique character, and it serves to illustrate and elucidate a number of points in connexion with recent advances in our knowledge of the pathology and etiology of enteric fever.

History of the Case.

Twenty-nine years ago Dr. W. T., a medical practitioner had as a young man, during a residence in the United States, a severe attack of typhoid fever. During the hungry stage of his convalescence, he suffered from dyspepsia on several occasions, and within three months of the period of his illness, he had an attack of very acute pain in the region of the liver, with a feeling of distension in the neighbourhood of the gall bladder.

Attacks of this character since recognized by him as biliary colic recurred about every three months for over a year. Since that time they have become less frequent, but every year one or more attacks occur, often during holidays, and are generally associated with over-exertion, long bicycle rides or walks, and irregular meals. Jaundice has sometimes been a feature of these attacks. His bowels act regularly, and his evacuations are normal. With the exception of these attacks, his health has been otherwise excellent, and he has carried on a very heavy practice during the last twenty years without being a single whole day off duty through ill-health. In view of the recent work on typhoid fever, it occurred to me that the case might be one of cholecystitis typhosa, and that W. T. was possibly a typhoid carrier. On discussing the question with him, he readily put himself at my disposal for investigation.

Bacteriological Examination.

In selecting the samples for plating, any mucus present in the faeces was included. A platinum loopful of faeces was emulsified in a broth tube containing 5 c.cm. of broth. Three loopfuls were then placed on the first of a series of Petri's plates containing MacConkey's bile-salt-lactose-neutral-red-agar. By means of a glass-rod the material was distributed over the surface of the medium, and the same rod was employed for the inoculation of the second and third plates of the series.

Three samples have been examined, and in all of them the typhoid bacillus has been demonstrated. The first sample gave only non-lactose fermenting colonies, all of which proved to be *B. typhosus*. The second sample gave about equal numbers of colonies of the typhoid bacillus and of *B. coli communis*, and in this case a few colonies of the *Bacillus faecalis alkaligenes* were also present. The third sample gave seventy colonies of the typhoid bacillus and one colony of *B. coli communis*.

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That we were here dealing with the typhoid bacillus was shown as follows:

The bacillus is a highly motile rod; it does not liquefy gelatine and does not produce indol. It produces acid, without gas formation, in litmus-peptone-water media containing glucose, laevulose, galactose, maltose, dextrin, and mannite, and no change in arabinose, saccharose, lactose, raffinose, inulin, erythrite, and dulcite; it produces acid in litmus milk.

The urine was examined, but no typhoid bacilli were found.

The bacillus is pathogenic for the guinea-pig. Three guinea-pigs were inoculated intraperitoneally with 0 5 c.cm., 1 c.cm., and 2 c.cm. respectively of a twenty-fours' culture in broth. The guinea-pig which received 2 c.cm. died within twenty-four hours, the other two animals survived.

The bacillus is agglutinated by an antityphoid horse serum in a dilution of 1 in 10,000.

Agglutination Tests with Patient's Serum.

With the bacillus ordinarily used in the Institute for the Widal test the patient's serum gave an agglutination in a concentration of 1 in 25, but failed to do so in 1 in 50, and 1 in 100.

With the patient's own bacillus the patient's serum failed to produce agglutination even in 1 in 25. That the absence of this reaction with the patient's own serum is not due to the lack of suitable receptors in the bacillus is proved by the fact that it was capable of being agglutinated by immune horse serum.

Within the last two years a revision of our conception of many of the factors concerned in the epidemiology and pathogenesis of typhoid fever has bocome necessary, and this has been almost entirely due to the work of Förster and his collaborators in the Strassburg laboratories. That work is being confirmed on all sides at the present time. Formerly we pictured the typhoid bacillus as finding its way into the alimentary canal, multiplying there, and invading the tissues, chiefly the lymphoid tissues, of the small intestine and the spleen, and only secondarily and fairly late in the disease finding its way into the blood stream.

The new conception, as outlined by Förster, is briefly as follows: The bacilli have been shown by Conradi to be present in the blood during the incubation period, and even before the bacilli can be found the presence of antibodies may be demonstrated by the precipitin test, as was shown by Fornet. On the other hand, it is only during the first and second weeks of the disease that the bacilli appear in the faeces. The bacilli, therefore, do not multiply in the gut, but at a very early stage find their way through the wall of the alimentary canal perhaps even from the fauces and tonsils into the lymphatics, and thence into the blood. While the bacillus is producing its effects on the lymphoid tissues of the gut, etc., other and not less important events, which till recently had been overlooked, are taking place.

At a very early stage the bacilli find their way into the liver and bile, and here their presence gives rise to inflammatory changes in the bile ducts and gall bladder. These changes have been observed in cases of disease in man, and also after the intravenous injection of the microbes into animals. The normal bile, according to Förster, is not a very suitable medium for the typhoid bacillus, but when albuminous material is added to it the bacillus finds in it an excellent pabulum. In the case of typhoid fever the albuminous material is supplied by the inflammatory exudate from the ducts and gall bladder, and the bacilli grow freely under these conditions in the gall bladder and pass out through the bile duct into the small intestine, thence into the large intestine, and are thrown out in the faeces. The irregular occurrence of the bacillus in the faeces is thus explained. In the later stages the bacilli from the ulcerated gut are added to those coming from the gall bladder.

In most cases recovery is accompanied by a cessation or clearing up of the inflammatory processes in the liver, bile ducts, and gall bladder; but in a certain number, about 2 per cent., of the cases this cholecystitis typhosa becomes a chronic process. Such cases are those which constitute the group of typhoid carriers. The bacillus continues to multiply in the gall bladder for periods varying from months to years, and to be thrown out into the gut and thence into the outer world. The gall bladder of such cases may be regarded as a normal habitat of the typhoid bacillus.

In certain of these cases gall stones occur, and it is still an undecided point whether these precede the typhoid fever, in which case they would play a part in the production of the typhoid carrier, or, as seems more probable, are the result of the processes indicated.

It is interesting to note that just as three-fourths of the cases of biliary calculi occur in women, so three-fourths of the typhoid carriers are also women.

That such typhoid carriers are, especially under certain conditions, a public menace has been proved by the investigation of many outbreaks. When the carriers are cooks, bakers, or in any way connected with the food supply, it has been conclusively demonstrated that they may act as the distributors of infection to many individuals. The importance of this question of typhoid carriers from the point of view of preventive medicine can hardly be overestimated. Such cases probably account for the sporadic cases the origin of which has, in the absence of this knowledge, been so puzzling. It also gives a key to the understanding of outbreaks of enteric fever among soldiers in the field. It is obvious that the first step in dealing with such cases is their recognition. This might be arrived at by systematic examination of the stools of all convalescents, and the knowledge of being a source of danger to others would in the case of many lead to greater care of the personal hygiene. I do not propose to enter into the discussion of this side of the question, as it has already been dealt with in this JOURNAL in a communition by Drs. A. and J. C. G. Ledingham.[1]

We have in this case, therefore, a typhoid carrier, and everything in the history points to the conclusion that the condition had its origin in the attack of typhoid fever which occurred in America twenty-nine years ago.

The suspicion that this was a typhoid carrier was not aroused by the occurrence of cases associated with the carrier, as has been the case in most of the isolated carriers hitherto investigated, but was deduced from the history, because, so far as Dr. T. knows, not a single case of infection originating from him has occurred. The fact that he is a bachelor with a small household has probably some influence in this, but it is counterbalanced by the fact that he has frequently lived in households containing from six to twelve individuals. This points to the conclusion that with educated people in fairly hygienic surroundings, and where personal hygiene is carefully attended to, the risk of infecting others is not so great as might be imagined. The very definite history of the first attack of biliary colic in a young man occurring three months after typhoid fever, strongly suggests that the calculi were the result of the infective processes.

The case raises many questions of the deepest interest from the point of view of immunity and bacteriology generally. That typhoid bacilli has been passing probably almost continuously for this long period from his gall bladder through the greater part of the alimentary canal, yet without causing recrudescence of the disease, and without producing any marked deleterious influence on the health, is itself a sufficiently striking fact.

In the majority of such cases which have been examined, there is evidence that by a process of absorption of the bacilli or their products immune substances are formed, and are thrown into the blood, the serum acquiring marked agglutinating properties.

In this case, however, the serum does not possess more agglutinins than are frequently present in the blood of healthy individuals, which suggests the possibility that here the absence of bad effects may be due to a local immunity. The fact that serum possesses such low agglutinating properties that it would have been reported as a "negative Widal" by most laboratories is an important point to be noted in the investigation of such cases, for it shows that a negative reaction does not exclude the possibility of a case being a typhoid carrier.

Another point of great interest is the extraordinary change which may take place in the bacterial flora of the faeces. A comparison of the results of plating the faeces in this case with those obtained under parallel conditions from normal cases, shows that there is an enormous reduction in the total number of organisms present in the faeces, and that the normal inhabitants have been altogether, or largely, replaced by the typhoid bacillus. Of the mechanism by which this is brought about we have no knowledge. It is hoped that the further investigation of this and similar cases may yield interesting results.

REFERENCE.

[1] A. and J. C. G. Ledingham, Typhoid Carriers, BRITISH MEDICAL JOURNAL, January 4th, 1908.

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No 15

SECTION V.

CHAPTER XIV.

THE TYPES OF IMMUNITY.

BY G. DEAN.

Introduction and definition. "Virulence and toxicity." Natural immunity. (*a*) Natural immunity in the individual, (*b*) Racial immunity, (*c*) Natural immunity in the species. Protective mechanism at the usual sites of diphtheria infection. Acquired immunity, active and passive. Inherited immunity.

Definitions and Introduction.

IMMUNITY may be defined as a condition of natural or acquired resistance possessed by individuals, races or species of animals to noxious influences which, in the absence of this refractory state, are capable of producing disease. The most important of these noxious influences are the animal and vegetable parasites, and chief among these the bacteria and their toxins.

Immunity is the expression of the reaction of the organism by which it attains partial or complete protection, and this varies according to the nature of the attack. In approaching the question of immunity, therefore, in any particular bacillary disease one of the first points for consideration is the method of attack of the invading microbe.

Though the pathogenic bacilli produce their effects on the animal organism chiefly by their toxins, the manner of the attack may vary greatly in the different groups of microbes. Diseases can be roughly classified according to the mode of action of the causal pathogenic micro-organisms, and such a classification is of the highest importance from the point of view of immunity.

One group of micro-organisms remains localised at or near the point of invasion. Here they elaborate their toxins, which are absorbed along the lymphatics, blood vessels or nerves, and find their way to and attack that part of the body for which they have a selective affinity. In such cases the predominant feature of the disease is the toxic action and the condition is known as a "toxaemia." Diphtheria and tetanus may be taken as types of this toxaemic form of disease.

In another group of bacterial diseases the microbe, having found an entrance into the animal body, does not remain localised but invades the blood, organs, and tissues of the body and multiplies in them. Anthrax and streptococcal septicaemia may be taken as types of this septicaemic group. There are other diseases which occupy an intermediate position between the two groups; of these typhoid and Malta fever may be mentioned as examples.

The organisms giving rise to the two types of disease differ from each other in one important respect. While those which cause the toxaemic type, when cultivated, freely secrete their poisons into the nutrient media, the others do not secrete soluble toxins. In fact it is difficult or impossible to obtain the toxins apart from the bodies of the latter bacteria.

The mechanism of immunity in the two groups is very different. In both cases the body strives to get rid of the invading parasite, but in the one group the chief reaction is directed against the toxic substances, which are being poured into the body; in the other group, the chief forces of resistance are directed against the microbes themselves.

The Terms "Virulence" and "Toxicity."

The *virulence* of a microbe is the power which it possesses of attacking, invading and breaking down the defences of a susceptible animal, thus giving rise to disease or death.

Toxicity is the power of a toxin to produce disease or death in a susceptible animal. The measure of the virulence or toxicity is inversely proportional to the quantity of living culture of the microbe or of its toxin required, under standard conditions, to produce a certain pathological effect; in the case of diphtheria, death within four days.

The toxicity of a culture or toxic filtrate is largely dependent on the toxigenicity of the race of micro-organism employed. The toxigenic property must be sharply differentiated from the virulence. The virulence of a micro-organism is no doubt largely due to its power of producing toxin within the animal body, but the toxigenicity of a particular microbe, as ascertained by measuring the toxicity of culture filtrates, cannot be taken as a true index of virulence. A race of diphtheria bacilli may have the property of producing toxin on artificial media very highly developed, and yet when introduced into the animal body may be less capable of producing pathological changes than a race with very low toxigenic properties.

NATURAL IMMUNITY.

Definition.

The property of resistance or insusceptibility possessed naturally by individuals, races, or species of animals to certain diseases is termed "natural immunity."

Examples of Natural Immunity with Remarks as to Mode of Origin and Causes.

(*a*) *Natural immunity in the individual.*

That certain individuals prove less susceptible than others when submitted to the same risk of infection is a matter of common knowledge. It is generally admitted also that an infective disease proves more serious to certain individuals than to others of apparently equal physical endurance. In cases of food poisoning, where a number of persons partake of about the same amount of infected material and thus get approximately the same dose of the infective germs, it is not an uncommon experience to find that one or two individuals of a series of say twenty, prove almost or altogether refractory to the infection. The causes of such immunity in individuals can only be surmised. Reference will be made to the subject in speaking of the question of antitoxin in the blood of normal individuals (p. 453).

(*b*) *Racial immunity.*

That the processes concerned in evolution are at work in the production of racial immunity, as in every other domain of biology, cannot be doubted. The benign character of certain diseases, such as measles, as they occur in an old established habitat among Europeans compared with their malignancy when imported into fresh regions, such as the

Sandwich Islands, can be most readily explained on the hypothesis of an immunity evolved by attacks of the disease affecting many generations. The dark races in Brazil are more refractory to yellow fever than the Europeans. How far these, and such similar examples as malaria, may be due to increased resistance as the result of evolution, and how far to an immunity acquired by the survival of the individual from an attack during childhood is unknown.

An excellent example of racial immunity among the lower animals is the comparative insusceptibility of the Algerian sheep to anthrax, one of the most deadly diseases to other races of sheep.

(c) *Natural immunity in the species.*

Metchnikoff (1901) in his review of the animal kingdom from the point of view of immunity found that most of the lowest forms of animal life were insusceptible to the action of diphtheria toxin. Among the insects he found, however, that while certain species were highly susceptible, other species, for example the larvae of a beetle (*Oryctes nasicornis*), manifested a marked immunity to the diphtheria bacillus and its toxin. The alligator (*A. mississippiensis*), while refractory to tetanus toxin, was found to be highly susceptible to diphtheria toxin.

An animal may possess a natural immunity to a bacillus, or to its toxins or both, but the possession of the one property does not necessarily imply the possession of the other. This distinction between immunity to infection and immunity to intoxication must be insisted on.

The rat and the mouse may be taken as examples of the lower animals possessing a natural immunity to diphtheria. These animals are capable of supporting very large doses of living cultures, or of toxin, when injected subcutaneously or intravenously. This immunity is not absolute, for, although the rat will support a dose of toxin containing many multiples of the lethal dose for the guinea-pig, by massive doses this resistance may be broken down, as was shown by Cobbett (1899) and others. That this natural immunity of the rat is not due to the presence in the blood of substances of the nature of antitoxins was demonstrated by Kuprianow (1894) who mixed rat's serum with toxin and found that the latter was not neutralised as evidenced by tests on guinea-pigs. Behring (1899) suggested that such cases of natural immunity are due to a physical cause, an impermeability of the walls of the capillaries which hinders the toxins from readily reaching the sensitive cells. Behring holds, however, that in certain cases one must assume a cellular immunity. But it can no longer be maintained that the immunity is in all cases due to an insusceptibility of the cells, for Roux and Borrel (1898) have shown that when the toxin is introduced directly into the cerebral substance it causes a paralysis, which may last several days and prove fatal. The toxin therefore, according to these authors, is innocuous not because the brain cells are insusceptible but because the toxin does not reach them. According to Metchnikoff this is due to the fact that the leucocytes set up a barrier which prevents the toxin from reaching the sensitive elements. The immunity of the rat and mouse may be explained in two ways from the standpoint of Ehrlich's side-chain theory. Either the animal may be wanting in the necessary side-chains by means of which the toxin is anchored to the sensitive cells, or the sensitive cells with suitable receptors may be distributed widely throughout the animal body, and in that way the force of the toxin may be diffused and diverted from organs necessary to the life of the animal. As a matter of fact no very satisfactory explanation has been given of the great differences which exist amongst the various species of animals in their susceptibility to toxins. In the chapter on diphtheria toxin will be found a statement of its relative toxicity for different species.

The question arises as to how far in the human subject the resistance possessed by certain individuals may be due to the presence of antitoxin in the blood. That antitoxin frequently occurs in the blood of healthy individuals has been shown by a number of observers, among them Abel (1894) and Wassermann (1894–5). The frequent presence of diphtheria bacilli in the throats of apparently healthy individuals (see p. 181) would yield a satisfactory explanation in the case of the human subject. Wassermann came to the conclusion that the occurrence of the antitoxin was due to the presence of "pseudo-diphtheria bacilli" in these cases. He thought that these organisms might produce small quantities of toxin sufficient to stimulate the antitoxin-producing mechanism, and yet not powerful enough to produce toxic effects. An observation by Lubowski (1900) is interesting in this connection. He isolated from a child a bacillus which proved to be avirulent and atoxic, and yet the serum of the child was found to be highly antitoxic. We must, however, distinguish between avirulent diphtheria bacilli and the bacillus of Hofmann. Petrie (1905) found that the latter was unable to produce any substances capable of giving rise to diphtheria antitoxin when injected into the horse.

Metchnikoff (1901) has suggested a mechanism which may account for the antitoxic properties of the blood serum of the hedgehog, snake, mongoose, and scorpion, namely, that these animals in fighting and catching their prey must frequently be subjected to slight abrasions with resulting absorption of small doses of toxin thus giving rise to the production of antitoxin.

It is more difficult to explain the presence of diphtheria antitoxin in the normal horse for here diphtheria bacilli are probably of rare occurrence. In one case only has their presence been definitely proved (Cobbett, 1900). Ehrlich explains the occurrence of antitoxin in the serum of normal animals by supposing that the antitoxin exists normally in a preformed state in the cells as the receptors which have a specific affinity for the toxin, and that some of these get thrown off into the blood serum. This subject will be dealt with more fully in discussing Ehrlich's theory.

Animals, which on account of their susceptibility to infection and intoxication are selected by preference for experiment, such as the guinea-pig and mouse in the case of anthrax and tetanus, and the guinea-pig in the case of diphtheria, may yet be regarded as immune to these diseases from the epidemiological, "epizootic" standpoint, for the occurrence of tetanus, anthrax and diphtheria in these animals under natural conditions is unknown. As Behring (1901, p. 95) has pointed out, some confusion may arise if the distinction between these two forms of immunity, the epidemiological and the experimental, is overlooked.

Protective Mechanism at the Usual Sites of Diphtheria Infection.

When we consider the number of organisms, often including pathogenic forms, such as the diphtheria bacillus, which find their way into the nose and mouth, it becomes clear that there must be at work an effective mechanism which prevents the settlement and growth of the invaders. That the conditions found in the alimentary canal from the mouth downwards are not unfavourable to microbial life is shown by the large number of non-pathogenic bacilli which can be cultivated from almost any part of its course, and as the pathogenic bacilli are not less resistant than the saprophytes we must assume that the conditions are favourable to many of them. What are the factors then which tend to prevent such pathogenic bacilli as the *B. diphtheriae* from attacking the individual? It is doubtful whether the antagonism between pathogenic and non-pathogenic bacilli plays an important part. Certain feeding experiments carried out by Metchnikoff (1894) and Wiener (1896) indicate that such an antagonistic action may occur. So long as the kittens and young rabbits, which were employed in the experiments, were being suckled by the mother they could be infected by certain vibrios *per os*, but as soon as they were put on a mixed diet infection could no longer be accomplished. The converse fact that non-pathogenic bacilli may aid the pathogenic has, however, been proved by many observers.

The epithelial layers, including the ciliated nasal epithelium of the mucous membranes, form an important barrier to the attack of microbes, and the constant shedding of this epithelium must be of great service in overcoming surface invasion. The secretions, mucus and saliva in the mouth, mucus in the nose, and tears in the conjunctival sac and nasal duct, have an important mechanical cleansing action. Particles of dust, etc., loaded with microbes, alighting on the sensitive mucous membrane cause an increase in the secretions which tends either to wash away the foreign substances, or by accumulating to give rise to the stimuli necessary for the reflex actions of coughing or sneezing, or the voluntary clearing of the nose or throat, all of which lead to the ejection of a large number of microbes. The muscular movements connected with the mouth, fauces, palate, and posterior nares must also aid in a mechanical way. The evidence as to whether the fluids concerned have bactericidal properties is somewhat conflicting. Wurtz and Lermoyez (1893) found the nasal mucus bactericidal, an opinion which was controverted by Klemperer (1896). Thompson and Hewlett (1896), though they found that the mucus was not bactericidal, proved that the nasal mucus arrested bacterial development and that there was a rapid disappearance from the nasal cavities of artificially introduced bacilli such as *B. prodigiosus*. In regard to the saliva Hugenschmidt (1896) found no bactericidal action, whereas Sanarelli (1891) found that it had bactericidal properties. As pointed out by Miller (1892) the evidence relied on by Sanarelli was insufficient. The important function exercised by the lymphoid tissues of this region has been urged by Metchnikoff, who regards them as manufactories of phagocytes. Stöhr (1884) made the important observation that the leucocytes wander out of the tonsils, and amongst others Ribbert (1885), Bizzozero (1885), Manfredi (1886), and Ruffer (1890) have shown that the cells of the lymphoid organs may contain bacilli. Every one who has examined the nasal secretion in pathological conditions must have been struck by the frequent occurrence of leucocytes containing bacilli. It is probable that the serum, though present only in minute traces in the secretions, may assist in preparing the bacilli for phagocytosis, and so play an important part in the defence of the body. Hugenschmidt (1896) attributes the protection of the mouth against invasion to phagocytosis aided by the positive chemiotactic character of the saliva. He maintains that diapedesis is a very marked feature in the mouth, and states that the sulphocyanide of potassium to which antiseptic properties had been ascribed has no such properties, and even doubts the existence of such a substance in saliva.

In the protection of the lungs several of the factors already mentioned play a part. That the phagocytes are as important here as elsewhere is probable. Hildebrandt (1888) found anthrax bacilli in the dust-cells, "Staubzellen," which he regards, however, as cast-off epithelial cells and not as macrophages, as held by Metchnikoff.

The general conditions which predispose to infection will be dealt with in another section. It is well, however, here to recall how susceptible the diphtheria bacillus is to slight changes in its surroundings, e.g., we shall see how the presence of an excess of sugar or a slight change in reaction of the culture medium can prevent toxin formation. Such considerations enable us to understand how very slight alterations in the animal body may have an important bearing in determining whether the bacillus will succeed or fail in obtaining a hold on the individual subjected to infection.

ACQUIRED IMMUNITY.

A susceptible animal may during its life acquire an immunity to a disease; in many cases an increase in natural resistance occurs as the animal grows older. From the earliest times it has been a matter of common observation that those who survived an infectious disease such as smallpox, syphilis, etc., acquired an immunity to that disease, in other words that recovery from a disease may do away with the natural proclivity to that disease. Where such an immunity is acquired by the survival of the individual from an attack of infective disease it may in a sense be regarded as "natural." It is Nature's way of conferring immunity, and the recognition of this fact has been the chief guiding principle in the study of the subject. On the other hand, where the immunity is acquired through the intervention of man by the employment of some process of inoculation, the immunity is termed "artificial." An artificial immunity may be conferred by the use of a bacterial or other vaccine, or by the use of an immune serum, and to designate these two conditions Ehrlich introduced the terms "active" and "passive" immunity, which may be thus defined—

Active immunity is the immunity which an animal acquires as the result of treatment with bacilli, toxins, or other substances, which may be of bacterial, vegetable, or animal origin.

Passive immunity is the immunity which results when an animal is injected with the antitoxin or other antibody, which has been obtained by the active immunisation of another animal.

In active immunity the immunised animal manufactures its own antibody. The immunity acquired by the horse as the result of injections of toxin may be taken as an example of active immunity. In passive immunity the immunised animal obtains its antibody ready made from another animal. The immunity conferred on a child by an injection of diphtheria antitoxin will serve as an example of passive immunity.

Behring (1899) takes exception to the terms "active" and "passive" immunity (p. 997). He says one can speak of an "active" or "passive" immunisation but he prefers to use the terms "isopathic" and "antitoxic" immunity. In both active and passive immunity he says that the essential thing is a haematogenous immunity which is due to the presence of antitoxin in the blood. It has still to be proved that there exists, in addition to this haematogenous immunity, a histogenous immunity due to an altered condition of the organised parts of the body, a view to which he formerly leant. There is, however, he admits, no sure experimental proof of the existence of an acquired histogenous immunity even in the case of an isopathic immunity, and it is now generally agreed that after the injection of antitoxin the living organised tissues remain unaffected, i.e., no histogenous immunity results. The immunity which a young animal acquires from its mother is derived from the milk and not by the inheritance of an acquired cell change.

Behring believes that, instead of increasing in their resistance, the cells susceptible to the action of toxin undergo changes due to modified activity which may result in a hypersensibility. If a hyposensibility could be demonstrated to result from active immunisation it would definitely prove the existence of a histogenous immunity. Behring at first thought such a hyposensibility could be observed in horses immunised against tetanus, but further observations made him abandon this view, for when the same antitoxic value of the blood serum had been produced in two animals, in one by passive, and in the other by active immunisation, the injection of equal doses of toxin appeared to produce more serious effects on the actively immunised animal than on the other. This he regarded as evidence of a hypersensibility. The observation that a horse which has been actively immunised retains his immunity much longer than a passively immunised man, guinea-pig, rabbit, or goat cannot be employed as an argument in favour of an increased resistance of the cells, which before immunisation were sensitive to the action of a toxin, for it has been found that if the serum of the same species is employed, the antitoxin remains much longer in the body than if the serum of another species is injected (Behring, Ransom).

This, however, is not universally true, for Jörgensen and Madsen (1902), in their study of cholera and typhoid agglutinins, found that the rapidity with which the antibody disappeared depended on the animal employed, and that no general law could be established. They found, for example, that the agglutinating serum of the rabbit and goat disappeared at about the same rate from the goat, whereas different results were obtained with the two sera in the case of the rabbit. In the same paper they make what appears to be a fundamental distinction between active and passive immunity. The injection of typhoid and cholera organisms was followed both in the normal animal and in the actively immunised animal by a sudden increase in the agglutinin giving a curve of a very definite type, whereas in the animal passively immunised there was an absence of this typical rise in the curve. The injection of quite a small quantity of agglutinating serum into an animal was sufficient to abolish the typical reaction.

INHERITED IMMUNITY.

Acquired immunity, obtained by the action on the body of the virus, whether introduced by Nature in the form of an attack of the disease or by man in the form of a vaccine, or by the injection of the serum of an immunised animal, is as a rule a transitory property belonging to the individual only. In certain cases, however, the immunity can be transferred from the parent to the offspring and to such cases the term "inherited immunity" is applied. Such an inherited immunity might conceivably be conveyed by

(*a*) the transference from the parents to the offspring of the acquired property by means of the germ plasm, (*b*) active immunisation; the toxin acting through the body of the mother on the germ plasm or on the developing foetus,

(*c*) passive immunisation; the foetus obtaining the protective substance manufactured by the body of the mother through the placental circulation or from the mother's milk.

The results of the experimental investigation of the relative importance to be ascribed to these possible sources of inherited immunity will be dealt with in a later section.

CHAPTER XV.

THE TOXIN OF THE DIPHTHERIA BACILLUS.

The term "toxin." Discovery of diphtheria toxin. Toxicity of different races of diphtheria bacilli. Conditions which modify the virulence and pathogenic powers of the diphtheria bacillus (*a*) association with other germs, (*b*) passage through animals. Influence of the medium on the production of toxin. Influence of temperature, oxygen, and light on the diphtheria bacillus. The period of maximum toxicity in the cultures. Methods of testing the toxicity of the diphtheria bacillus. The relative susceptibility of different races of animals to diphtheria toxin. On the origin and nature of toxin. The constitution of the diphtheria toxin. Toxoids. The spectrum according to Ehrlich. Conditions which modify toxin, time, light, temperature, electricity, oxygen, peroxides and oxydases. Chemical agents. The purification and isolation of the diphtheria toxin. The effects of toxin on animals. Subcutaneous injection. Latent period. Local effect. General effect. Intracerebral injection. Physiological effects on the heart and circulation. The fate of the toxin *in corpore* (1) in susceptible animals, (2) in refractory animals, (3) behaviour of toxin in the alimentary canal, (4) action of toxin on other mucous membranes.

The Term "Toxin."

TOXIN, in the restricted bacteriological sense, may be defined as the specific poison secreted by a pathogenic micro-organism. By the specific poison is meant the substance giving rise to the chief pathological effects which taken together constitute the disease.

The true toxins can be recovered from the cadaver of a subject of the disease and also from the media in which the bacilli have been grown.

The bacilli from which such toxins have been obtained constitute a small group, the chief of which are *Bacillus diphtheriae, Bacillus tetani, Bacillus botulinus* and *Bacillus pyocyaneus.*

The action of a toxin on the animal body is characterised by (*a*) the occurrence of a latent or incubation period after the injection, during which no effect on the animal is apparent, and (*b*) the production of antitoxin.

The toxin is a "haptine," that is, a substance capable of anchoring onto certain structures of the body and stimulating them to the formation of an antibody.

We have no exact knowledge as to the chemical nature of toxins. Though generally associated with albuminous substances, they are not at the present time usually regarded as toxalbumins. They are unstable, being extremely sensitive to the action of light, heat and chemicals. In many of their characteristics they closely resemble the ferments.

The word toxin in the wider sense has been applied to various poisonous bodies elaborated by micro-organisms including the "ptomaines," or crystalline nitrogenous bases of definite chemical composition, the toxalbumins and the proteins, substances entering into the structure of the bacillary body, none of which merit the title of toxin in the narrower sense as they are not concerned in producing the specific effect of the bacillary action. Even such substances as tuberculin and mallein, though they produce certain specific reactions, can be spoken of as toxins only in a broad sense.

The term toxin is also applied to various poisons of animal and vegetable origin; as examples of the former may be mentioned the snake poisons, or venins, and the ichthyotoxin of eel's serum; of the latter such substances as ricin and abrin.

DISCOVERY OF DIPHTHERIA TOXIN.

Loeffler (1884) in his work on the diphtheria bacillus indicated that it was necessary to assume the existence of a toxin to explain satisfactorily the production of the disease by the bacillus. At a later period (1890) he was able to prove that a toxin could be obtained by growing the bacillus on minced beef, extracting with glycerine, and precipitating by alcohol.

To Roux and Yersin (1888, 1889, 1890) belongs the credit of first obtaining a diphtheria toxin. By methods which could easily be followed and repeated they obtained toxins which gave all the characteristic appearances of diphtheria poisoning. The essential point in the method consisted in growing the diphtheria bacillus on broth for several weeks at body temperature and filtering the culture through an unglazed clay filter. The toxicity of sterile filtrates from cultures 30 to 40 days old was such that guinea-pigs were killed in about 30 hours by doses of from $\frac{1}{8}$ to $\frac{1}{15}$ c.c. Though the toxins obtained were weak compared with those that can be obtained at the present time their action on animals, and the effects on the toxin of heat, chemical agents, dialysis, etc., were so accurately noted and described that the memoirs of Roux and Yersin on the subject must always rank as among the most noteworthy of the classics of experimental medicine.

Toxicity and Virulence of Different Races of Diphtheria Bacilli.

When cultures are made from cases of diphtheria the bacilli, grown under the same conditions on a medium suitable for toxin production, yield filtrates of very different degrees of toxicity. It cannot be said that there is any very marked correspondence between the severity of the disease in the human subject and the virulence or toxigenic power of the bacillus obtained from the case. Some of the most virulent and toxigenic races of bacilli have been derived from comparatively slight cases of diphtheria. A notable example of this is Park and Williams' bacillus No. 8 which was obtained from a mild tonsillar case. As a rule the most virulent bacilli yield the strongest toxins but not invariably. A bacillus which has a low degree of virulence may yield a stronger toxin than a highly virulent one (Behring (1901), Salter (1896)). Indeed, as shown by Martin (1898) a bacillus may be toxigenic and yet non-virulent. A process of selection, therefore, has been employed to obtain bacilli which yield powerful toxins. As the race of bacillus is, perhaps, the most important factor involved in obtaining good toxins, a great service was done by Park and Williams who obtained a very highly toxigenic race which is now used all over the world.

The bacilli obtained from cases of diphtheria have as a rule a toxicity which can be expressed by stating that from 0·1 ($\frac{1}{10}$) c.c. to 0·02 ($\frac{1}{50}$) c.c. of the filtrate of a ten days' old culture on alkaline broth injected subcutaneously into a guinea-pig of 250 grammes weight kills the animal within five days; rarely 0·01 ($\frac{1}{100}$) c.c. produces this result. By employing the American bacillus under the same conditions toxins can frequently be obtained which give the above-mentioned effect in doses of from 0·005 to 0·003 c.c. and occasionally 0·002 c.c. or even smaller doses (Madsen, and personal experience).

Conditions which modify the Virulence and the Toxigenic Power of the Diphtheria Bacillus.

Various methods have been employed to obtain an elevation of the virulence and of the toxigenicity of the diphtheria bacillus.

(*a*) *Through association with other germs.*

Roux and Yersin (1890) and Funck (1894) observed that when the diphtheria bacillus and the streptococcus were both found in the throat there was a tendency for the type of infection to be severe. On this observation and certain experiments the hypothesis was based that the bacterial association led to an increase in the virulence of the bacillus. By inoculating rabbits with diphtheria bacilli of low virulence together with highly virulent streptococci they obtained bacilli which retained the newly acquired property on re-inoculation in pure culture. The influence of association on these two micro-organisms has been much investigated, the subject having been approached from various points of view.

Von Dungern (1896–7) did not find that the association of these germs in cultures gave rise to more vigorous growth, whereas Bernheim (1897) and Hilbert (1898) both held that the growth tended to be more rapid and abundant. Hilbert also found that association with the streptococcus gave rise to a more rapid and increased production of alkali and toxin. In many cases where cultures of the diphtheria bacillus were remaining at a standstill, in the atoxic acid phase, the addition of streptococci led to alkali and toxin formation. Hilbert discusses the cause of this increase of toxicity. He points out that rapid growth does not necessarily indicate rapid toxin formation, for Blumenthal (1897) showed that in media to which glucose had been added, vigorous and rapid growth of the diphtheria bacillus took place, while at the same time toxin formation was completely inhibited. Escherich (1894) attributed the rise in virulence obtained in the experiments of Roux and Yersin to passage through the animal body. Hilbert, though believing that the virulence is increased by association, ascribes a considerable part of the increased toxicity to an alteration in the medium, produced by the growth of the streptococci. A filtrate from a streptococcal culture proved an excellent medium for the growth and toxin production of the diphtheria bacillus. Gibier (1897) suggested the association of the streptococcus with the diphtheria bacillus as a practical method of obtaining a powerful diphtheria toxin. He found that if the streptococcus were allowed to grow 12 or 18 hours in a broth, before introducing the diphtheria bacillus, the resulting medium was favourable to the growth and toxigenicity of the diphtheria bacillus.

(*b*) *By passage through animals.*

Bardach (1895) used passage through dogs to elevate the virulence. Trumpp (1896) injected avirulent bacilli together with toxin and found that the bacillus recovered from the dead animal was sufficiently virulent to kill a guinea-pig. Hewlett and Knight (1897) believed that they succeeded in converting Hofmann's bacillus into the diphtheria bacillus by heating the cultures and also by passage through guinea-pigs. Salter (1899) claimed to have obtained the same result by passage through small birds. Louis Martin (1898) cultivated the bacillus within the peritoneal cavity of rabbits in the collodion sacs, which were first employed by Metchnikoff, Roux and Salimbeni in their study of cholera. By the passage of the bacillus in this manner through several rabbits a marked increase in toxigenic power was acquired, e.g., after six passages a bacillus, which formerly gave a toxin killing guinea-pigs at $\frac{1}{10}$ c.c. now gave a toxin killing at $\frac{1}{50}$ c.c. By the same treatment he was able to augment the toxigenic power of the American bacillus though the passage had not increased the virulence. Many of the previous experimenters made no distinction between toxigenicity and virulence, but Martin makes a clear distinction between the two properties.

An interesting point to note in connection with the toxigenic property of the diphtheria bacillus is the persistence with which certain races maintain this character. Park and Williams' toxigenic bacillus, though it has been cultivated on artificial media for 11 years, appears to have suffered no diminution in its toxigenicity.

The Influence of the Medium on the Production of Toxin.

For obtaining powerful toxins the character of the medium is most important. Roux and Yersin (1889–1900) employed simple broth and obtained only weak toxins. Later it was found that the addition of peptone to the broth gave better results. The influence of the amount of peptone was studied by Park and Williams (1896).

They found that as a rule the addition of 2 % and 4 % of peptone gave more powerful toxins than the addition of 1 %. The writer failed to find that 4 % peptone broth gave more powerful toxins than 2 %. The quality of the peptone is probably most important; certain preparations appear to give more powerful toxins than do others, under apparently similar conditions. Witte's peptone is the one in common use.

The reaction of the broth is of great importance.

The growth of the diphtheria bacillus in ordinary broth gives rise to various changes in the reaction. At first the broth becomes slightly alkaline, then there is an acid phase occurring in three or four days. The acid phase may persist or the reaction, after a few days, may become alkaline.

The relation of this acid phase to the presence of glucose in the beef was investigated by Spronck (1895), van Turenhout (1895), Park and Williams (1896), Cobbett (1897), and Blumenthal (1897). They found that where glucose was present except in very small quantities the acid phase was very marked and tended to be permanent. The persistence of the acid phase is deleterious to the growth of the bacillus and to the production of toxin. Madsen (1897) found that the formation of toxin and the degree of alkalinity run parallel, but that while a high degree of alkalinity is usually associated with a high toxicity this is not invariable. Cultures which have an acid reaction when filtered, say after ten days' growth, are atoxic. These observations show the importance of avoiding a marked or persistent acid phase, and many suggestions have been made in this direction. Spronck and van Turenhout (1895), attributing the excessive production of acid to the presence of glucose, recommended that beef which has been kept several days, during which decomposition of the glucose takes place, should be employed. They also suggested the addition of calcium carbonate to the medium, so that when the acid is formed it should enter into combination with the alkaline earth and in this way the medium would remain alkaline, thus aiding the formation of toxin. Nicolle (1896) in opposition to Spronck recommends that the meat should be fresh. Theobald Smith (1899) finds that dextrose is not in itself inimical to toxin production, but that a certain quantity is in fact essential to an abundant accumulation of toxin. He gets rid of the muscle sugar from the beef infusion by inoculating this with a culture of *B. coli* and incubating overnight, but afterwards adds to his medium a measured quantity, about 0·1 % of dextrose. In this way he states that he obtains a greater uniformity in the toxicity of the filtrates.

Park and Williams find that if the medium is made sufficiently alkaline the amount of glucose usually present even in the fresh (two days old) unfermented meat is not sufficiently great to interfere with toxin production. After bringing the medium to the neutral point of litmus they add from 5 to 10 c.c. of normal caustic soda per litre. The writer's personal experience coincides with this.

Martin's medium has been used in the attempt to obtain a medium of more constant composition which would give more uniformly filtrates of a high degree of toxicity. A peptone solution is obtained as follows: the mucous membrane and muscle of five pigs' stomachs are minced and mixed with water acidulated with hydrochloric acid. The proportions are as follows :

Pigs' stomachs, minced	200 grammes
Hydrochloric acid, pure	10 c.c.
Water	1000 c.c.

Digestion is allowed to proceed at 50° C. and is normally completed in 12 hours, but no harm is done by allowing the digestion to go on for 24 hours as no organisms develop in the acid mixture. The mixture is heated to 100° C. which destroys the remaining pepsin, and is then passed through a sieve, or, better, filtered through a thin and loosely packed layer of absorbent cotton wool. The medium is then heated and made alkaline while at 80° C. It is then filtered through paper to remove the precipitate, heated to 120° C., filtered and put in culture flasks in which it is sterilised by heating for half an hour to 115 °C.

This medium by itself is suitable for cultivating organisms, but better results are obtained by mixing it with broth. The peptone prepared in this way gives more constant results than the very variable peptones of commerce.

The broth is derived from veal. After testing the value of fermenting with a yeast to get rid of the glucose Martin came to the conclusion that the best results were obtained by allowing the minced veal to ferment without using any special organism for the purpose. Five hundred grammes of minced veal are mixed with 1,000 c.c. of water and put in the incubator for 20 hours at 35° C., ·5 $^0/_0$ of sodium chloride is added, the fluid made alkaline, and then sterilised. Equal parts of this and the peptone solution are now heated to 70° C., to coagulate the albuminous substances, filtered through paper, made alkaline and sterilised. The sterilisation which gives the best result according to Martin is filtration through a Chamberland filter, but good results are obtained with sterilisation at 120° C. Slight variations under certain conditions are employed. Toxin is obtained by this method which kills a guinea-pig of 500 grammes weight in a dose of $\frac{1}{50}$ c.c.

Martin's medium gives excellent toxins, but in the writer's experience a uniform high toxicity is not attained by this nor by any other process. The use of a boiled and filtered extract of bakers' yeast with the addition of 2 $^0/_0$ peptone as was recommended by Spronck (1898) gave disappointing practical results.

The writer's best results, confirmed by Madsen and Dreyer, have been obtained by the employment of the following method, which is somewhat similar to that described by Park and Williams, and is less prolonged and complicated than that of Martin.

The beef which is employed is that sold by English butchers under the name of "silverside," consisting of part of the muscles of the gluteal region. The meat is used either in a perfectly fresh condition or after it has been kept about seven to twelve days in an ice-cooled air chamber at about 8° C. It has never been frozen nor does it come in contact with the ice. This beef is freed from all fat and fascia and is then finely minced in a mincing machine. Usually 10 to 20 litres of the broth are made at one time. The proportion of beef and water is about one pound (about half a kilo) of beef to 1 litre of a tap water which is fairly alkaline from the presence of lime salts. Appended will be found an analysis of this water.

Chemical Examination.

Determinations	Results in Grains per gallon	Results in Parts per 100,000
Chlorine	1·3	1·8
Equivalent to chlorides (60 °/$_0$ Cl) ...	2·1	3·0
Nitric nitrogen	0·28	0·4
Equivalent to nitrates (17 °/$_0$ N) ...	1·68	2·4
Nitrites	Absent	Absent
Hardness: permanent ,, temporary	5°	—
Lead, copper, zinc, iron	Absent	Absent
Free ammonia	0·0040	0·0057
Organic ammonia	0·00028	0·0004

The beef and water are then put into a large enamelled ordinary cooking pot with a loose cover and the mixture is kept boiling quietly for 1½ to 2 hours. It is then filtered through Swedish filter paper, the juice at the same time being very thoroughly squeezed out from the beef. A potato or vegetable squeezer is very convenient for this purpose. To the filtrate so obtained are added 2 °/$_0$ Witte's peptone and 0·5 °/$_0$ sodium chloride. The liquid is then steamed at 100° C. for one hour, and the resulting precipitate is filtered off. At this stage, and while still hot, the filtrate is made neutral to litmus paper, and then 7 c.c. of normal caustic soda are added per litre. It is again steamed for one hour and again filtered. The broth is then put in the culture flasks, usually Erlenmeyer's, and sterilised by steaming for 20 minutes, the temperature being then allowed to run up to 120° C., at which point the gas burner is turned off.

A number of experiments performed on the influence of temperature in the preparation and sterilisation of this broth showed that the temperature employed had little or no influence on the end result.

The toxicity for guinea-pigs of 250–280 grammes weight is shown in the Tables A and B which give the results on two continuous series of toxins obtained by the use of this broth. These include all the toxins prepared and tested during that period and are not selected.

TABLE A.

Diphtheria Toxins—September, 1899, *to June,* 1902.

Minimal lethal dose and time of death in days.

Toxin in c.cs.	Days:—1	2	3	4	5	6	6+
1/500	—	—	—	—	2	—	—
1/400	—	3	8	2	—	1	—
1/300	—	2	8	3	3	1	2*
1/200	—	3	5	4	3	2	5*
1/100	—	10	6	3	—	2	14*
1/50	—	1	—	—	—	—	—
1/30	—	—	—	—	—	—	1

* Not tested in larger doses.

TABLE A—(*continued*).

Total number of toxins, 94.

2 killed within 6 days at 1/500, i.e. 2 °/
14 ,, ,, ,, 1/400 ,, 15 °/₀
17 ,, ,, ,, 1/300 ,, 18 °/₀
17 ,, ,, ,, 1/200 ,, 18 °/₀
21 ,, ,, ,, 1/100 ,, 22 °/₀
16 did not kill at 1/100 ,, 17 °/₀

TABLE B.

Diphtheria Toxins from January, 1904, *to June,* 1905.

Minimal lethal dose and time of death in days.

Toxin in c.cs.	1	2	3	4	5	6	6+
1/700	—	—	—	—	—	1	—
1/600	—	—	—	1	—	—	—
1/500	—	1	5	—	1	1	—
1/400	—	2	2	2	1	2	—
1/300	—	1	5	—	1	—	—
1/200	—	1	2	1	3	—	5*
1/100	—	1	—	—	—	—	—

* Not tested in larger doses.

Total number of toxins, 39.

1 killed within 6 days at 1/700, i.e. 2·6 °/₀
1 ,, ,, ,, 1/600 ,, 2·6 °/₀
8 ,, ,, ,, 1/500 ,, 20·5 °/₀
9 ,, ,, ,, 1/400 ,, 23·1 °/₀
7 ,, ,, ,, 1/300 ,, 18·0 °/₀
7 ,, ,, ,, 1/200 ,, 18·0 °/₀
1 ,, ,, ,, 1/100 ,, 2·6 °/₀
5 did not kill at 1/200 ,, 13·0 °/₀

Besides the race of bacillus and the medium employed there are other important factors which must be considered in connection with the preparation of diphtheria toxin.

(*c*) *Influence of temperature, oxygen and light on the diphtheria bacillus.*

The supply of oxygen.—To obtain abundant growth and good toxin formation a free supply of oxygen is required.

Roux and Martin (1894) suggested the use of Fernbach's flat flasks over which a current of air could be passed by the use of a water pump. Madsen (1896) found on comparing the toxins which were obtained by growing the bacillus in Erlenmeyer's flasks and in Fernbach's flasks with an air current, that the latter method gave weaker toxin, an experience that coincides with the writer's. This is probably due to excessive oxidation of the toxin and to a disturbance of the membrane on the surface of the broth. Though artificial aeration is unnecessary and the form of the culture flask immaterial, it is essential (as was pointed out by Aronson (1894)) that the medium should form a thin layer so that a large surface may be exposed. The amount of material used for inoculation has been suggested as a possible cause in the variation of the toxicity of the different flasks of one series. The best results have been obtained by the writer by inoculating from one broth flask to another. A flask with a good membrane is selected, and a 2 millegramme loopful of membrane is gently floated onto the surface of the medium to be inoculated.

It is now generally agreed that the optimum temperature for the growth of the diphtheria bacillus and for toxin production is 35·5° C., a little below body temperature.

Light must be rigidly excluded from the incubator, or *chambre-étuve*, as it has a deleterious action on the growth of the bacillus.

Period of Maximum Toxicity.

The views regarding the time in which the maximum amount of toxin is produced in a culture have greatly changed. Roux and Yersin (1890–1894) stated that in cultures grown without aeration a strong toxin was not produced under three weeks' incubation, but that when a constant air current was employed two weeks' incubation was necessary. Aronson, however, showed that by inoculating from the pellicle of a highly virulent culture he could obtain a potent toxin in eight days. It is now generally held that the maximum toxicity is reached about the tenth day (Aronson, 1895; Park and Williams, 1896), the time varying between the 5th and the 12th day. Recently Murillo (1903) has pointed out that a secondary rise of toxicity occurs about the 24th day, which he holds is probably due to a liberation of an intrabacillary toxin.

The above tables are formed from rough estimations of the minimal lethal dose, usually expressed by the letters M. L. D. When the M. L. D. is being ascertained for the purpose of determining the constitution of a toxin the doses are more finely graded. For example, if, by a rough test, a toxin has been found to kill a 250 gramme guinea-pig in a dose of about $\frac{1}{300}$ c.c., it is desirable to grade the series of test doses as follows:—0·0028, 0·0029, 0·003, 0·0031, 0·0032, 0·0033, and 0·0034. The smallest dose killing on the fourth or fifth day is regarded by Ehrlich as the M. L. D. The selection of this time-limit is quite arbitrary.

Arrhenius and Madsen (1904) have introduced two new methods by the use of which they assert that it is possible to form a more correct estimation of the M. L. D. than by the use of direct valuation only. One is based on the loss of weight, and the other on the time which elapses between the injection of toxin and the death of the animal. By utilising these two methods many experimental data which would otherwise be useless, become valuable for calculation[1].

Loss of Weight.

There is usually a progressive loss of weight during the first few days after the injection, which reaches its maximum on the fifth or sixth day. From a very large number of experiments with fresh toxins the average loss of weight per day has been calculated. If the maximum loss on the fifth day is represented by 100 the average loss at the end of each day is shown in the following table.

1.	2.	3.	4.	5.	6.	7.	8.	10.	14.	days.
40	52	70	87	100	98	94	78	51	11	average loss of weight.

Having ascertained the loss of weight on any of the first four days after injection of the toxin, it is possible by means of these ratios to obtain a calculated value for the maximal loss of weight on the fifth day. A definite relation is held to exist between such maxima and the dose of toxin.

Calculated maximal loss of weight in grammes :—	120	100	80	65	60	55	50	45	40	35	30	20	10	0	<0
Dose :—	1·30	1·20	1·10	1·00	0·92	0·85	0·78	0·70	0·61	0·55	0·50	0·45	0·40	0·35	0·35

Time of Death.

In a similar manner from a large number of experiments they find that if the animal dies in

1 day	the	dose	corresponds	to	1·6	minimal	lethal	doses.
1·5 days	,,	,,	,,		1·4	,,	,,	,,
2 ,,	,,	,,	,,		1·25	,,	,,	,,
2·5 ,,	,,	,,	,,		1·15	,,	,,	,,
3 ,,	,,	,,	,,		1·05	,,	,,	,,
3·3 ,,	,,	,,	,,		1·0	,,	,,	,,
3·5 ,,	,,	,,	,,		0·97	,,	,,	,,
4 ,,	,,	,,	,,		0·91	,,	,,	,,
4·5 ,,	,,	,,	,,		0·85	,,	,,	,,
5 ,,	,,	,,	,,		0·80	,,	,,	,,

formaı

enius and Madsen find that the data obtained by the two methods approximate

Roux aı

Craw and Dean (1907) have recently examined a large number of data from

current of aiıt.

Their results do not altogether support the deductions of Arrhenius and

on comparing

meyer's flasks a

6 days the dose corresponds to 0·71 minimal lethal doses.
7 ,, ,, ,, ,, 0·64 ,, ,, ,,
8 ,, ,, ,, ,, 0·59 ,, ,, ,,
9 ,, ,, ,, ,, 0·55 ,, ,, ,,
10 ,, ,, ,, ,, 0·51 ,, ,, ,,
12 ,, ,, ,, ,, 0·45 ,, ,, ,,
14 ,, ,, ,, ,, 0·4 ,, ,, ,,
0·4
>14 ,, ,, ,, ,, <0·4 ,, ,, ,,

The Relative Susceptibility of different Species of Animals to Diphtheria Toxin.

The relative susceptibility of animals to toxins is usually recorded in terms of the quantity of toxin required to kill a gramme weight. The guinea-pig, being the animal most frequently employed for experimental work on diphtheria toxin, is usually taken as the standard. The guinea-pig is as a rule wonderfully uniform in its resistance but Ehrlich and Theobald Smith have recorded racial variations in susceptibility, and the English guinea-pig is stated to be more resistant than the German. Ehrlich has found the susceptibility of the pigeon and the guinea-pig to be so nearly equal that it has been possible to use pigeons for test animals, when there has been a dearth of guinea-pigs. Behring (1899) found the rabbit twice as sensitive as the guinea-pig; other observers put them as nearly equal. The rabbit is much less susceptible than the guinea-pig to infection with the living bacillus. The horse requires only a fraction of the guinea-pig's lethal dose per gramme to kill him, and the goat has a similar high susceptibility. The more resistant rat, dog, cow and sheep require several multiples of the guinea-pig's lethal dose. White mice are very resistant requiring as much as 1,000 times the guinea-pig's lethal dose.

Calabrese and Zagari (1895) stated that cold-blooded animals were insusceptible, but Courmont, Doyon and Paviot (1895) found that frogs injected with diphtheria toxin, and kept at high temperatures such as 30° C., lost flesh and became paralysed. Reference has already been made to the susceptibility of the alligator (p. 452).

On the Origin and Nature of the Toxin.

On account of its enzyme-like qualities, Loeffler looked upon diphtheria toxin as a secretion. Gamaleia (1892; quoted by Kossel) thought that the toxin originated by the solution of the toxic bodies of the bacilli, i.e., by a form of autolysis or lixiviation. In favour of this view he adduced the fact that, notwithstanding great bacterial growth, practically no toxin could be found in the culture fluid separated from the bacilli during the first few days of their growth. Guinochet (1892) and Ouschinsky (1893), who attempted to solve the question of the origin of toxin by growing the bacilli in proteid-free nutrient media, came to the conclusion that the toxin was produced by a synthetic process within the bacterial cell. As, however, their results in regard to diphtheria were not confirmed by Fränkel (1894), Hugonnency and Doyon (1896) or Doyon (1896), little or no growth being obtained on their media, much weight cannot be attached to their deductions. Kossel (1896) demonstrated that even a one day old culture contained toxin in the broth. By means of a 1 % solution of caustic soda he succeeded in extracting toxic substances from the bodies of the bacilli killed by chloroform. From a great quantity of bacilli obtained by three days' growth in ten flat flasks, he extracted 10 c.c. of a clear, light yellow fluid which gave no reaction with Millon's reagent, and only a trace of Biuret reaction. This fluid in doses of 0·4 c.c. killed guinea-pigs in less than 48 hours with all the signs of typical diphtheria intoxication.

In consideration of these facts, namely, the rapid appearance of toxin in the nutrient medium, and the very small quantity of toxin obtainable by solution of the bacilli, Kossel concludes that the production of toxin cannot be attributed to a mere autolysis of the toxic bodies of the bacilli. He assumes, therefore, that the toxin is elaborated from the nutrient substances by a metabolic process within the body of the bacillus and is excreted.

Brieger and Boer (1896) found that after the removal of the last traces of soluble toxin from the bacillary bodies (which was best effected by washing with a concentrated ammonium chloride solution), the bodies of the bacilli still contained a highly toxic substance which was characterised by its power of causing necrosis, by its thermostability, and by the fact that its action was not neutralised by antitoxin. These results have frequently been confirmed.

Vicquerat (1902), in a paper with somewhat fanciful views, holds that there are four phases in the diphtheria culture:—

1. Formation of lactic acid by the fermentation of the carbohydrates,
2. Ammonia and para-lactic acid formation from peptone,
3. Solution of the diphtheria bacilli,
4. Toxin formation.

Murillo (1903) from his observation of the secondary rise in the curve of toxin formation (see p. 469) believes in the existence of an intrabacillary toxin without having definite views as to whether maceration, autolysis, or lixiviation is the factor at work in setting it free.

Rist (1903) recovered the bacilli from the filter papers used in filtering cultures during the preparation of diphtheria toxin. The bacilli were heated for 24 hours with a mixture of alcohol and ether, dried *in vacuo* over sulphuric acid, and kept *in vacuo* in the dark. He found, in accordance with Brieger and Boer's results, that antitoxin did not preserve the life of the animal against bacilli treated in this way and made into an emulsion with salt solution. He is inclined to attribute the paralysis, myocarditis, and late death generally, to the action of this intrabacillary toxin.

Notwithstanding these views the writer believes that the trend of opinion amongst experienced workers is to regard toxin formation as due not to a degeneration or death of the bacilli but to a genuine process of secretion.

THE CONSTITUTION OF DIPHTHERIA TOXIN.

The greater part of our knowledge of the structure of diphtheria toxin is due to Ehrlich. Realising that the ordinary filtrate from a diphtheria culture was a very complex body, and that there seemed little probability of obtaining a toxin in a pure state by chemical means, Ehrlich proceeded to investigate the subject from a biological standpoint. He examined for this purpose twelve crude toxins, making for each certain quantitative determinations. The absolute toxicity was accurately measured by ascertaining the exact dose required to kill a guinea-pig weighing 250 grammes on the fourth or fifth day. This is called the minimal lethal dose (M. L. D.) and may be regarded as the unit of Toxin (T). The neutralisation of the toxin by means of an arbitrary quantity or unit of serum was next tested and two limits were fixed which were of great importance in determining the constitution of the toxin[1].

The quantity of toxin which is neutralised by one antitoxic unit is designated by Ehrlich "L_0" (limes nul = Glattwert). The mixture of L_0 and one unit of antitoxin is completely without effect on animals. L_+ (limes Tod = death limit) is the smallest quantity of toxin which, when mixed with one antitoxic unit, causes the death of the guinea-pig within five days. Now if diphtheria toxin were a simple chemical body, $L_+ - L_0 = (D)$ the difference should be, when expressed in lethal doses, equal to one lethal dose. That is, the addition to the L_0 of one M. L. D. or T, should give the L_+ which causes the death of the guinea-pig. To illustrate these points one may take a theoretically pure toxin of which the M. L. D. or T is 0·01 c.c., and the L_0 1 c.c. Then one would find that as T = 0·01 c.c.

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[1] The antitoxic unit, = "A. U." (I. E. = Immunitätseinheit, Ehrlich) will be dealt with in a later section, but may be taken here as the quantity of antitoxin which is contained in 1 c.c. of a serum of which 0·1 c.c. neutralises the tenfold M. L. D. for a guinea-pig. An A. U. is then capable of neutralising 100 M. L. D.

$$\begin{array}{ll} L_+ = 1{\cdot}01 & = 101\ \text{T.} \\ L_0 = 1{\cdot}00 & = 100\ \text{T.} \\ \hline \text{D (difference)} & = 0{\cdot}01 = 1\ \text{T.} \end{array}$$

Such a state of affairs is, however, never found. To convert the mixture of L_0 and 1 A.U. into the L_+ one must always add more than 1 T. Ehrlich ascertained by working out these constants in the 12 toxins specially investigated that the difference between the L_+ and the L_0 was never a single lethal dose; it varied from 1·7 to 28 minimal lethal doses. Now of these 28 M. L. D. only one is required to kill the animal, the others appear to have been fixed by the antitoxin in such a manner that they produce no effect.

From this it is clear that the absolute toxicity of a toxin and its power of neutralising antitoxin do not stand in direct relation to one another. There must therefore be other non-lethal substances in the crude toxin which have the power of entering into combination with or neutralising antitoxin. On the assumption that a fixed quantity of toxin will always enter into combination with the same quantity of antitoxin, and having regard to the specific relation of the antitoxin to the toxin, the natural deduction is that these non-lethal antitoxin neutralising substances are derivatives or modifications of the toxin, and with this idea Ehrlich calls them "toxoids." One of the strongest proofs of the existence of toxoids is obtained from observing the changes which occur on keeping diphtheria toxins. It is frequently found that while the quantity of toxin neutralised by one unit remains the same there takes place a progressive diminution in the absolute toxicity of the poison. Ehrlich, for example, found that the L_0 value of a toxin was 0·31, and that at first its M. L. D. for guinea-pigs was 0·003. Nine months later the M. L. D. was 0·009, that is, it had only one-third of its original toxicity, whereas its combining power with antitoxin remained unchanged, the L_0 still being 0·31. In this case although the combining power of the toxin had remained unaltered two-thirds of the toxin had become converted into toxoids.

The toxoids, as a rule, are present only in small quantities in fresh cultures; it is only in old cultures or filtrates which have been kept a long time, or which have been subjected to the action of chemical agents, that they are present in large quantity. The toxin molecule may be regarded as possessing a stable, combining or "haptophorous group" (ἅπτω = I grasp) and an unstable "toxophorous group." The bearing of this on Ehrlich's theory of antitoxin production is important and will be dealt with later.

If the toxoids are responsible for the large number of lethal doses in D, it must be due to their having different affinities to antitoxin. Looked at from this point of view of affinity to antitoxin, three groups are conceivable:—

(1) Protoxoids, which possess a greater affinity than the toxin,
(2) Syntoxoids, which have an affinity equal to the toxin, and
(3) Epitoxoids, or toxones, which have an affinity less than the toxin[1].

Ehrlich assumes that the toxin and antitoxin in their combination follow the law of equivalents, and that a toxin unit which has been converted into a toxoid unit is still capable of combining with the same amount of antitoxin.

If protoxoids are present together with toxin in a filtrate and antitoxin is gradually added, the protoxoids, owing to their great avidity for antitoxin, must be saturated before any of the antitoxin will enter into combination with the toxin. Ehrlich uses as a rough chemical analogy the phenomena which occur on the addition of an alkali, analogous to antitoxin, to two acids of different combining affinities, for example, hydrochloric acid, analogue of the protoxoid, and acetic acid, analogue of the toxin. If, for instance, a diphtheria filtrate contains 99 parts of protoxoid and one part of toxin and is completely neutralised by antitoxin, the state of affairs may be represented by the following equation:—

$$\overbrace{99\ \text{protoxoid-antitoxin}} + \overbrace{1\ \text{toxin-antitoxin}} = \text{physiologically neutral.}$$
$$99\ \text{NaCl} + 1\ \text{acetate of soda} = \text{chemically neutral.}$$

If one adds to the mixture one equivalent of protoxoid, or, in analogous fashion, one equivalent of hydrochloric acid, the re-arrangement resulting may be represented thus:—

$$100\ \text{protoxoid-antitoxin} + 1\ \text{free toxin} = L_+$$
$$100\ \text{NaCl} + \text{acetic acid} = \text{free acetic acid};$$

---

[1] Epitoxoids and toxones. At first Ehrlich believed that the substance with least affinity to antitoxin was like the two others, an altered toxin, and therefore named it epitoxoid, but later the results obtained by the examination of three fresh toxins forced him to the conclusion that it was a primary secretion of the bacillus, and to mark this change of view he adopted the name of toxone in place of epitoxoid.

the elements with the greater affinity combining and leaving those with less affinity uncombined.

If one adds to an L_0 mixture of toxin and syntoxoid further quantities of the original mixture of toxin and syntoxoid, the union of the latter is not influenced by the addition of toxin, and one finds that death results when the added quantity contains a toxic unit. The presence of protoxoids or syntoxoids then does not raise the value of D, since the addition of one toxin equivalent is at once evident.

The influence of the presence of epitoxoids, or toxones, is very different. If one adds toxin equivalents to a mixture containing toxones, the former all seem to disappear without immediate effect, and the toxic action is only shown after sufficient toxin equivalents have been added to use up all the antitoxin previously occupied by the toxone. This is seen in the scheme :—

90 toxin-antitoxin + 10 epitoxoid-antitoxin = physiologically neutral.

If one adds to this 11 toxin units one obtains

100 toxin-antitoxin + 1 toxin free + 10 epitoxoid free = L_+.

From a consideration of these facts, the reason why the difference, D, between the L_0 and the L_+ is not a single lethal dose becomes evident. It is the toxone alone which is responsible for this elevation of the value of D.

The characteristics of the epitoxoids, or toxones, may be ascertained from a study of the effects produced on guinea-pigs by the mixtures of antitoxin and toxin lying between the L_0 and the L_+ doses, namely doses in which the toxin is not completely neutralised, and is yet not present in sufficient quantity to cause the death of the animal on the fourth or fifth day. The effects in this zone must be due chiefly to toxone action since it is here that the toxones, displaced by the toxin from their union with the antitoxin, become free.

Non-lethal doses of toxin, containing from half to three-fourths of a lethal dose, when injected subcutaneously into guinea-pigs produce very constant results characterised by the occurrence of extensive infiltration, at first soft but afterwards becoming indurated, followed by necrosis of the skin and alopecia: paralysis with toxin alone is rare.

Now in the guinea-pigs receiving toxin-antitoxin mixtures between the L_0 and the L_+ doses the infiltration is, as a rule, softer and tends to disappear without necrosis and alopecia. Even when the infiltration has been slight and has rapidly disappeared paralysis is a frequent occurrence, the onset taking place usually about the third week. So common indeed is paralysis under these conditions that Ehrlich regards the toxone as the element in the crude toxin which is alone responsible for the paralysis.

The progressive loss of toxicity in a number of toxins showed an interesting arithmetical relation. Ehrlich points out that a number of diphtheria toxins are so constituted that one antitoxic unit completely neutralises about 100 toxic units, that is, the L_0 dose corresponds to about 100 toxic units. On the other hand, in the majority the L_0 dose contains a smaller number than this, and the number, allowing for experimental error, has the relation to 100 of $\frac{1}{2}$ or $\frac{1}{3}$.

Thus Ehrlich found the following numbers

47·5, 54·4, mean 50·9.
33, 32, 33·2, 33·4, 35·7, mean 33·4.

From these experiments it may be argued that:—

(1) Toxins lose their toxic powers, in simple proportions,
(2) The power of fixing the antitoxin is constant,
(3) In many cases one antitoxic unit combines with a quantity of toxin representing 100 toxic units.

These facts compel us to assume that the L_0 of these toxins originally contained 100 T, but that a conversion of the toxin into toxoids occurred in the simple proportion of 1 : 2 or 1 : 3.

In determining the antitoxic value of his standard serum Ehrlich used a toxin which contained equal parts of toxin and toxone. One hundred M. L. D. of that poison therefore contained 100 equivalents of toxin and 100 equivalents of toxone, and the quantity of serum which neutralised 100 lethal doses in reality fixed 200 equivalents of the poison. He concluded that the combining power of the antitoxic unit was 200, and that this unit would always combine with 200 equivalents of all diphtheria toxins whatever proportion of toxin, toxoids and toxones they might contain.

If this neutralising power of one unit is accepted the following formulae can be deduced when

a represents the number of units of toxin contained in L_0.
Z ,, ,, ,, ,, ,, toxone ,, ,,
and $\beta = D - 1$.

Then $200 - Z =$ the amount of toxin and toxoid,
$200 - a - Z =$ the amount of toxoid,
$L_0 = (200 - Z)$ toxin-toxoid $+$ Z toxone,
$L_0 = (200 - a - Z)$ toxoid $+$ a toxin $+$ Z toxone $=$ 200 equivalents.

Adding 1 unit antitoxin.

1 unit antitoxin $+$ $L_0 = (200 - a - Z)$ toxoid-antitoxin
$+$ a toxin-antitoxin $+$ Z toxone-antitoxin.

To set one toxone free, since we have 200 − Z equivalents of greater affinity, of which there are a toxin equivalents, it is necessary to add to L_0 $\frac{1}{200 - Z}$ of which $\frac{1}{200 - Z} \cdot a$ is the number of toxin equivalents.

To set Z toxone equivalents free it is necessary to add $\frac{Z}{200 - Z}$ of which $\frac{Z}{200 - Z} \cdot a$ are toxin equivalents, viz., the quantity of toxin solution expressed in toxic units which must be added to 1 antitoxin unit + L_0 to set free all the toxone equivalents which, with the addition of one toxin equivalent, produces the L_+ dose. That quantity may be designated β which is expressed by the difference between L_+ and L_0 minus 1 M.L.D., i.e., D − 1[1], and a may be obtained by dividing the L_0 dose by M.L.D. $a = \frac{L_0}{\text{M.L.D.}}$, so that

$$\beta = \frac{Z}{200 - Z} \cdot a,$$

$$\beta\,(200 - Z) = Za,$$

$$aZ + \beta Z = 200\beta,$$

$$Z = \frac{200\beta}{a + \beta}.$$

This may be more conveniently expressed in the following way[2].

Let Z = no. of toxone equivalents in the L_0 dose,

a = ,, toxin equivalents in the L_0 dose, i.e., toxin expressed as M.L.D.

Then each of the (200 − Z) toxin-toxoid equivalents may be considered to contain $\frac{a}{200 - Z}$ toxin equivalents.

Now if one adds an amount of toxin (P) expressed in M.L.D. which will bring the L_0 dose up to the L_+ dose − 1 M.L.D., the 200 antitoxin equivalents will now be able to combine with an amount of toxin = a + P.

$$\therefore \text{ each equivalent now contains } \frac{a + P}{200} \text{ toxin.}$$

But by theory Z of these equivalents must be exactly equal to the amount of toxin added,

$$\therefore Z\,\frac{a + P}{200} = P,$$

$$\text{i.e., } Z = 200\,\frac{P}{a + P}.$$

$$Z = \text{toxone} = 200\,\frac{(L_+ - L_0 - 1)}{L_0 + (L_+ - L_0 - 1)} = \frac{200\,(L_+ - L_0 - 1)}{L_+ - 1}.$$

---

[1] As shown by Park and Atkinson (1898) and Madsen (1899) there is an error in deducting 1. This would be correct only in dealing with a theoretically pure toxin: it should be a fraction of 1.

[2] I am indebted to Dr J. C. G. Ledingham for this solution.

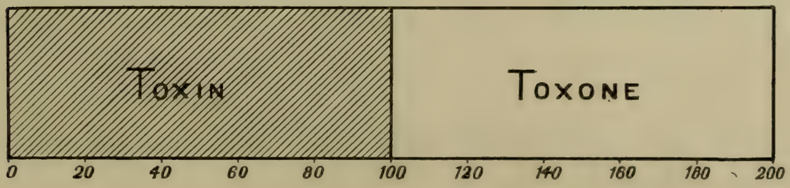
By calculating the number of toxone equivalents present in the L_0 dose of different filtrates one finds the numbers 25, 33, 33, 50, 66, 67, and 100. These numbers show a similar proportional relation to 200 as is observed in connection with the numbers of toxin equivalents.

Park and Atkinson pointed out that the neutralising value of a lethal dose of toxin is lowest when the first considerable amounts of toxin have appeared in the culture. After a short period as the toxin in the culture increases, the neutralising value of the fatal dose begins to increase at first rapidly and then more slowly. After the cessation of toxin production the neutralising value of the fatal dose increases till it may reach five to ten times its original amount. This is quite in accordance with Ehrlich's view of toxoid formation. They point out, however, that the changing of this limit, e.g. to the sixth or seventh day, would entirely alter the values of the L_+ and L_0 and, therefore, the results of his formula. They comment on the difficulty or impossibility of determining the L_0 dose. While refusing to admit the value of Ehrlich's formulae, they admit the great practical value of employing his antitoxic unit as a standard.

The Method of Partial Saturation.

A still more intimate knowledge of the constitution of a diphtheria toxin may be obtained by using the method of partial saturation of the toxin by antitoxin. This method has given most valuable results in the hands of Ehrlich and Madsen, on whose work this account is based. The results obtained depend upon the difference of avidity for antitoxin possessed by the various constituent parts of the toxin. To give a graphic record of these results Ehrlich has introduced a form of schema to which he gives the name of "Toxin Spectrum." Along the axis of the abscissa, which is divided into 200 equal parts representing the 200 equivalents of poison, he distributes the toxin constituents from the left to the right, putting to the left those with the greatest affinity, viz. the protoxoids, and to the right those which possess the least affinity, viz. the toxones. The shaded ordinates indicate the amount of pure toxin present at each particular part of the abscissa, and those unshaded show the amount of toxoid and toxone.

The L_0 of a toxin containing no toxoids but only pure toxin and toxone in equal parts, would be represented by the following figure.

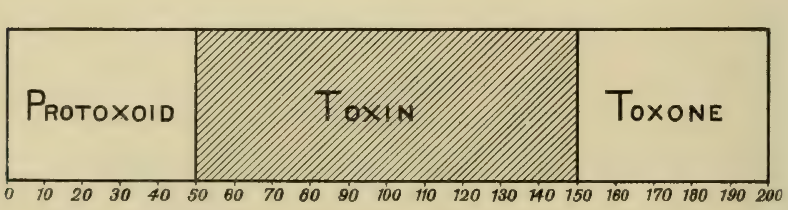


If in this case $\frac{200}{200}$ of an A.U. is added to the L_0 of toxin we have complete neutralisation. If $\frac{199}{200}$ of an A.U. is added, a small quantity of the element with the least affinity to antitoxin will be unneutralised, i.e. a certain amount of toxone. When $\frac{150}{200}$ of an A.U. is added only toxone remains unneutralised.

,, $\frac{100}{200}$,, ,, ,, ,, ,,
,, $\frac{99}{200}$,, ,, 1 lethal dose + the toxone remains unneutralised.
,, $\frac{98}{200}$,, ,, 2 lethal doses + the toxone ,, ,,
,, $\frac{60}{200}$,, ,, 40 ,, ,, ,, ,, ,,
,, $\frac{10}{200}$,, ,, 90 ,, ,, ,, ,, ,,
i.e., the mixture has enough toxin to kill 90 guinea-pigs.
,, $\frac{1}{200}$ of an A.U. is added 99 lethal doses + the toxone remains unneutralised, i.e., the mixture has enough toxin to kill 99 guinea-pigs.

A toxin of such simple structure as represented here has no actual existence.

The L_0 of a toxin containing 50 protoxoid equivalents, 100 toxin equivalents, and 50 toxone equivalents, will be represented thus:—



The L_0 + $\frac{200}{200}$ of an A.U. will not kill a guinea-pig nor produce symptoms.

L_0 + $\frac{199}{200}$ A.U. results in 1 toxone equivalent being free.

L_0 + $\frac{150}{200}$ A.U. results in 50 toxone equivalents being free, and does not kill one guinea-pig.

When a large number of toxone equivalents remain unneutralised we get a transitory oedema at the site of inoculation but not death. Paralysis and late death may follow.

With L_0 + $\frac{149}{200}$ A.U. the 50 toxone equivalents and 1 toxin equivalent are unneutralised and the mixture kills 1 guinea-pig.

Similarly L_0 + $\frac{148}{200}$ A.U. kills 2 guinea-pigs.
L_0 + $\frac{147}{200}$,, 3 ,,
L_0 + $\frac{100}{200}$,, 50 ,,
L_0 + $\frac{70}{200}$,, 80 ,,
L_0 + $\frac{50}{200}$,, 100 ,,

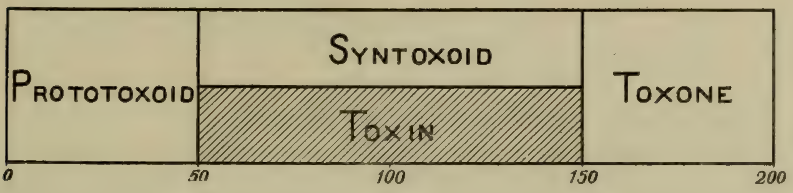
On the addition of lesser quantities than $\frac{50}{100}$ A.U., since all the available toxin is free, no more than the 100 guinea-pigs are killed. On the existence of this non-toxic zone is based the chief argument in favour of the existence of protoxoids.

L_0 + $\frac{50}{200}$ kills 100 guinea-pigs.
L_0 + $\frac{49}{200}$,, ,,
L_0 + $\frac{1}{200}$,, ,,

The facts may be stated, perhaps more clearly, in another way.

If we add to the L_0 dose which we know contains x lethal doses, $\frac{1}{200}$, $\frac{2}{200}$, $\frac{3}{200}$, $\frac{4}{200}$, and so on up to $\frac{50}{200}$ of an A. U. we do not find any diminution of the toxicity of these successive mixtures; each still contains x lethal doses. Up to a certain point we seem to have added the antitoxin in vain; it disappears in the mixture without reducing its toxicity. In the example taken this happens until we reach $\frac{51}{200}$ when the number of lethal doses is found to be $x - 1$, $\frac{52}{200} = x - 2$, and so on.

The same toxin may undergo a change, half the toxin being converted into syntoxoid; the dichotomous division or "hemitoxin-stadium" of Ehrlich. The L_0 now contains only 50 toxin equivalents:—

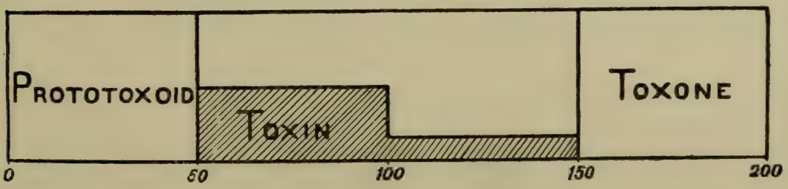


The toxone part of the spectrum will be found unaltered:

$L_0 + \frac{200}{200}$	kills	0	guinea-pigs.
$\frac{150}{200}$	,,	0	,,
$\frac{149}{200}$	,,	0	,,
$\frac{148}{200}$	,,	1	,,
$\frac{147}{200}$	,,	1	,,
$\frac{146}{200}$	,,	2	,,
$\frac{145}{200}$	,,	2	,,

It will be noted that now the mixture containing the L_0 dose and $\frac{149}{200}$ A. U. does not kill a guinea-pig, to do so the mixture must contain $\frac{148}{200}$ A. U., and to kill two guinea-pigs $\frac{146}{200}$ must be added, that is, to kill each additional guinea-pig, instead of diminishing the amount of antitoxin added by one antitoxin equivalent, it is necessary to diminish by two antitoxin equivalents, that is by $\frac{2}{200}$ A. U. instead of by $\frac{1}{200}$ A. U. In other words, half the substance using up the antitoxin is now atoxic.

If we assume a still further weakening of the toxin so that the L_0 dose contains now only 30 toxic units, the distribution may be represented thus:—



Then $L_0 + \frac{200}{200}$ kills 0 guinea-pigs.
$\frac{150}{200}$ „ 0 „
$\frac{141}{200}$ „ 0 „
$\frac{140}{200}$ „ 1 „
$\frac{145}{200}$ „ 1 „
$\frac{130}{200}$ „ 2 „
$\frac{125}{200}$ „ 2 „
$\frac{120}{200}$ „ 3 „
$\frac{100}{200}$ „ 5 „
$\frac{99}{200}$ „ 5 „
$\frac{98}{200}$ „ 6 „
$\frac{97}{200}$ „ 6 „
$\frac{96}{200}$ „ 7 „
$\frac{90}{200}$ „ 10 „
$\frac{50}{200}$ „ 30 „
$\frac{1}{200}$ „ 30 „

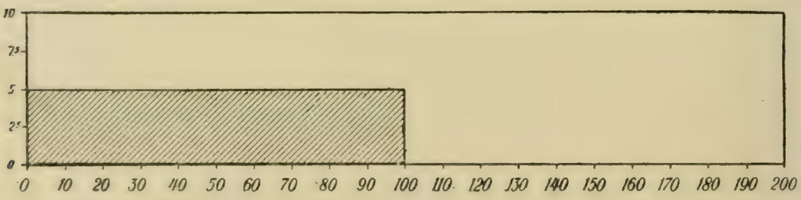
Neither the protoxoid nor the toxone zone is here altered. In the part of the spectrum from 150 to 100, the distribution of toxin and toxoid is such that to liberate one toxin equivalent it is necessary to liberate nine toxoid equivalents.

Ehrlich (1898–1903) has shown how this method may be applied to the study of the different phases through which a toxin passes. A toxin, No. 5, had at first the following constants :

$$L_0 = 0{\cdot}125 \text{ c.cm.}$$
$$L_+ = 0{\cdot}25 \text{ c.cm.}$$
$$\text{M.L.D.} = 0{\cdot}0025 \text{ c.cm.}$$

It will be therefore seen that the L_0 contained 50 M.L.D., and the L_+, being twice the L_0, contained 100 M.L.D.

The toxin had three distinct phases in so far as its toxin content was concerned.



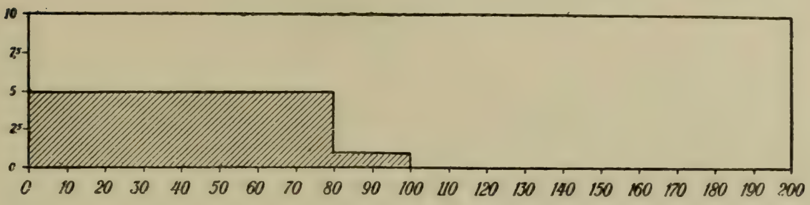
In the First Phase the toxin had the distribution shown in the above figure, that is, it showed a pure hemitoxin. Every antitoxin unit added to the L_0 dose reduced the toxicity by one half M.L.D., that is, by the addition of every $\frac{2}{200}$ A.U. the mixture was lethal to one guinea-pig less. This happened only during the addition of the first hundred $\frac{1}{200}$ A.U., the addition of more antitoxin only influenced the toxone action.

Where the hemitoxin zone of a toxin reaches in the L_0 mixture to 100 what will the L_+ dose be? The L_+ will be reached when 200 equivalents of hemitoxin are present and when one M.L.D. is left free ; then theoretically we must multiply the

L_0 by $\frac{202}{100}$ to get the L_+ for $\frac{L_+}{L_0} = \frac{202}{100}$. This gives the L_+ as 0·253 and the observed value is 0·25, a remarkably close agreement with the calculated value.

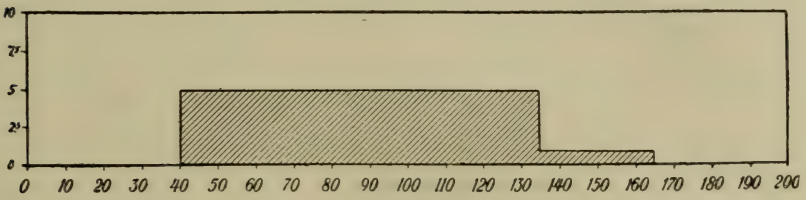
In the Second Phase the L_+ had increased to 0·26 and the M.L.D. had risen from 0·0025 to 0·003. The L_0 dose now contained 42 instead of 50 M.L.D.

The distribution of the toxin is represented in the following figure. From 80 to 100 only $\frac{1}{10}$ of the toxin now remains unaltered. If the fall in toxicity be due to a conversion of the end, or tritotoxin part of the hemitoxin, into toxoid so that only $\frac{1}{10}$ of the toxin is left unchanged, then to obtain the L_+ from the L_0 we must multiply the L_0 not by $\frac{202}{100}$ but by $\frac{210}{100}$. By this calculation we obtain the L_+ as 0·262, again a remarkable agreement with the observed value 0·26 "and a little over."



In the Third Phase there was no change in the toxin region but a loss of toxone had occurred which accounted for a change in the L_0 dose from 0·125 to 0·21.

In the Fourth Phase the L_+ was still ·26 but the M.L.D. had risen from 0·003 to 0·004 so that 22 M.L.D. had disappeared from the L_0.



As shown in the spectrum figure above the disappearance was due to the appearance of an extensive prototoxoid zone affecting the first 40 toxic units of the spectrum. No loss, however, in the combining units had taken place; the toxophorous group alone and not the haptophorous group was affected.

The schemes given will suffice to illustrate the method introduced by Ehrlich to record the constitution of toxins, but a simplicity of structure such as illustrated is never met with in practice. The toxins, and also probably the toxones, do not occur in the form of simple bodies, but fall into various sub-divisions which are differentiated by Ehrlich according to their avidity for antitoxin, so that in a descending scale we have proto-, deutero-, and tritotoxins.

Still further complications exist for we must assume that every one of these varieties of toxin consists of equal parts of two modifications, which have the same relation to antitoxin but differ from one another in their relations to external disturbing influences. The one, the α-modification, is in all toxins readily converted into toxoid. The second, or β-modification, varies in stability in the different groups as follows :

β-modification of deuterotoxin, very stable.
„ prototoxin, less stable.
„ tritotoxin, very unstable.

Although the conversion of the β tritotoxin into toxoid begins early it is never complete ; traces of toxin are always present in this part of the spectrum. Later the β-prototoxin disappears altogether. Finally the β-deuterotoxin remains with the trace of β-tritotoxin, and at this stage the changes cease and a stable condition ensues. The possibility of obtaining suitable constant test toxins depends on the stability of the β-deuterotoxin.

To explain certain phenomena Ehrlich had to assume the existence of toxonoids. Madsen and Dreyer (1901) found that a mixture which was completely neutral for guinea-pigs was still acutely toxic for rabbits, and that mixtures containing even less toxin than this L_0 mixture were capable of producing paralysis in rabbits. To obtain, therefore, the L_0 value for rabbits it was necessary to go considerably below the L_0 value for guinea-pigs.

From these results it appeared that toxin-antitoxin mixtures which showed typical toxone or paralysing action in one species, the rabbit, showed no observable action on another species, the guinea-pig, and further that what produced paralysis, or toxone action, in one species (the guinea-pig) produced toxin (acute lethal) action in another species (the rabbit).

Ehrlich (1903) explained these results by the assumption that there were three varieties of toxin, each having a different degree of avidity for antitoxin :—

(1) Toxin, with the greatest avidity, acutely toxic for rabbits and guinea-pigs and more toxic for the former,
(2) toxone, acutely toxic for rabbits, killing guinea-pigs with paralysis,
(3) Toxonoids harmless to guinea-pigs, causing paralysis in rabbits.

A further investigation of the question by Morgenroth (1904) has shown that the assumption of toxonoids, so far as concerns these experiments, is unnecessary. He found that the explanation of the results depends on two factors. Ehrlich, finding that no difference could be observed whether the mixtures were injected 15 minutes after the toxin was added to the antitoxin or after standing together 24 hours, came to the conclusion that the reaction was a rapid one and was completed within 15 minutes. This appears to be quite true only so far as subcutaneous injection into guinea-pigs is concerned. Dreyer and Madsen used in their experiments subcutaneous injection in the case of the guinea-pig and intravenous injection in the case of the rabbit. Now Morgenroth has found that when the toxin-antitoxin mixtures, which show only toxone action when injected subcutaneously into guinea-pigs, give rise to acute toxic action on intracardiac injection. He further found that if a long enough time, say 24 hours, is given for complete union to take place between toxin and antitoxin before the intravenous injection, the mixture, which is completely neutralised for the guinea-pig, is also neutralised for the rabbit.

CONDITIONS WHICH MODIFY TOXIN.

Time.

Toxins are very unstable substances, and a certain period must elapse after filtration before an equilibrium in their constitution is established. Even then their stability is very uncertain, and without any obvious cause they may within a short time undergo great alterations in their composition. The factors at work in producing the changes, which occur with the lapse of time, are very obscure and have eluded investigation. An analogy may be found in the difficulty of determining the exact processes which play a part in the maturing of a wine.

Several of the modifying factors to be discussed are no doubt concerned in the production of the changes.

Light.

Light, especially in the presence of oxygen, has a very deleterious action on toxin. A few days' exposure to diffuse daylight, or a few hours' exposure to direct sunlight, causes a marked deterioration. Piazza (1895) found that the red and violet rays (the heat and actinic rays) of the spectrum, were active, the yellow rays inactive.

Temperature.

Effect of heat on diphtheria toxin.

Exposure to a temperature of 58° C. for two hours, or 100° C. for 20 minutes, almost completely destroys the toxicity of filtrates. Toxin precipitated with calcium phosphate and dried is much more resistant; it withstands a temperature of 70° C., and is not much affected even by a temperature of 100° C. for 20 minutes. Behring holds that toxins heated for half an hour to 100° C. are still capable of producing certain toxic effects on goats and horses.

Effect of low temperatures.

Diphtheria toxin is unaffected by low temperatures. Sudden changes of temperature appear to be injurious.

Action of electricity on toxin.

Smirnow (1894–95) stated that by means of electricity toxin could be modified and converted into substances which acted like antitoxin. His results were obtained by the employment of a continuous current of low tension. Krüger (1895) by the same procedure obtained confirmatory results, while D'Arsonval and Charrin (1896) obtained similar results with alternating currents of high frequency.

For a brief period the hope was entertained that it might be possible to obtain antitoxins without the intervention of the animal organism. Such an idea had soon to be abandoned. Marmier (1896) investigated the subject in a critical and independent spirit, with the object of discovering whether the changes produced in toxin are due to the electricity itself or due to secondary phenomena, the result of electrical action. From his investigation he concluded that continuous or alternating currents of low frequency destroy or attenuate toxins by the production of hypochlorites or chlorine from the sodium chloride present in the toxin. A series of experiments performed by L. Martin, proved that the quantity of hypochlorites present in Smirnow's experiments was greater than was necessary to protect a guinea-pig from a lethal dose of toxin. Currents of high frequency have, apart from the heat evolved, not the slightest attenuating action on toxins.

Oxygen.

Oxygen also destroys toxin. Hence, toxin conserved under toluol retains its toxic properties better than toxin whose surface is in contact with the air. Brieger and Boer (1896) found it to be much more affected by oxidising than by reducing agents.

Peroxides and Oxydases.

Sieber (1901) found that diphtheria and tetanus toxin were inactivated by peroxides and by oxydases of animal and vegetable origin.

Action of Chemical Agents.

Acids. Roux and Yersin found that lactic and tartaric acids almost completely abolished the toxicity of the toxic filtrates. Boric acid, borates and phenol act in the same way, but less vigorously. On neutralising the acidulated toxins some of the original toxicity is recovered. Behring (1894) also found that the inorganic acids acted deleteriously on toxin. Nevertheless he observed that even 1 % hydrochloric acid left a considerable amount of toxin unchanged. More recently Ritchie (1901) has also observed that diphtheria toxin is capable of resisting the action of hydrochloric acid.

Alkalies. While Behring found that a 2 % solution of sodium hydrate had less influence on toxin than 1 % hydrochloric acid, Ritchie (1901) found that diphtheria toxin was very susceptible to its action.

Toxin modified by these means is still capable of producing immunity.

Behring found that toxin was unaffected by 1 % carbolic acid and also by other benzol derivatives and by pure iodoform and chloroform acting in the dark. The metal salts were also harmless, provided they did not act as oxidising agents or yield free chlorine or iodine. Only formaldehyde or iodine-trichloride had a greater effect than the acids. L. Martin observed that the hypochlorites acted injuriously on toxin, and Salkowski (1898) found that antiseptics in strong concentrations exerted a markedly destructive action.

The action of chemical agents on toxin is further dealt with in the section on the purification of toxin (p. 448).

Effect of Bacterial Growth on Toxin.

The growth of living germs, which have contaminated a toxin, exerts a markedly attenuating action. The rapidity and extent of the deterioration depend on the nature of the organisms, and their capacity to attack the different constituents of the broth. On this subject very little is known, but as a rule it is found that the growth of a mould has a much less injurious influence on a toxin than the growth of a bacterium. Nobécourt (1900) found that yeasts caused an attenuation of toxin.

The Purification and Isolation of Diphtheria Toxin.

All attempts at the isolation of diphtheria toxin in the pure state have ended in failure; the negative results, however, are valuable and interesting from the historical standpoint.

The first effort was made in this direction by Loeffler who by the addition of alcohol to a glycerine extract of a broth culture obtained a precipitate, easily soluble in water, which produced the pathological effects of the toxin. Roux and Yersin early observed that precipitates caused by the action of various substances, such as calcium phosphate, on toxic filtrates tended to carry the toxin down with them. Brieger and Fraenkel (1890) were the first to systematically investigate the subject, and as the result of their work concluded that toxin belonged to the serum albumin group and gave it the name of "toxalbumin." They obtained this substance by the use of such precipitating agents as ammonium sulphate, sodium sulphate, and alcohol. Their best results were obtained with alcohol, and their method was briefly as follows:—the broth filtrate, reduced by evaporation to one-third of its volume, was added to a ten-fold volume of absolute alcohol slightly acidulated with acetic acid. After the mixture had stood 12 hours in the ice-box, the precipitate was removed by filtration, re-dissolved in water, and again precipitated. This was repeated six to eight times, till the precipitate when dissolved in water gave a perfectly clear solution. The final precipitate was dialysed and dried *in vacuo* at 40° C. In this way a white powder was obtained which was not precipitated from its watery solution by boiling nor by the action of sodium chloride, sodium or magnesium sulphate, lead acetate or nitric acid, but gave all the other proteid reactions, including precipitation by carbonic acid. It was, therefore, closely allied to the albumoses. Its approximate percentage composition was 45·35 % carbon, 7·13 % hydrogen, 16·33 % nitrogen, 1·39 % sulphur, 29·80 % oxygen.

Wassermann and Proskauer (1891) employed an elaborate modification of this method.

Brieger and Boer (1896) carried out a number of experiments with the object of separating the toxins of diphtheria and tetanus from all other substances, such as the inactive albuminates to which they are bound on precipitation.

They succeeded in obtaining double compounds with zinc salts. The separation of the toxin from these compounds is, however, difficult or impossible on account of the ease with which it undergoes destruction in the presence of certain reagents, such as carbonic acid, sulphuretted hydrogen, sodium phosphate and various acids, which consequently are not available.

On the other hand, alkaline and neutral salts of the alkalis or alkaline earths, and especially certain ammonia salts are not markedly injurious and are thus suitable for the process. The method which gave the best results with diphtheria toxin was carried out as follows. Two volumes of 1 % zinc chloride solution were added to the sterile filtrate. The precipitate was washed and shaken with a volume of ammonium carbonate (3 to 6 %) equal to the original volume of the broth culture. Ammonium phosphate was added until solution of the precipitate took place and the supernatant fluid began to grow turbid owing to the presence of zinc phosphate. The deposit was allowed to settle and was removed by filtration. The filtrate was saturated with ammonium sulphate. The resulting precipitate contained the diphtheria toxin. The accompanying peptone was got rid of by filtration, after the repeated addition of finely powdered sodium sulphate. The toxin thus obtained gave no proteid reaction and was optically inactive.

Wassermann and Proskauer and Brieger and Boer succeeded by their methods in obtaining the toxic substance from the organs of animals that had died of diphtheria intoxication.

A substance of very great physiological activity is not obtained by any of the methods which have been described. During the manipulation a great loss of toxin occurs.

THE EFFECT OF THE TOXIN ON ANIMALS.

(*a*) *Subcutaneous Injection.*

Latent period. When a lethal dose of toxin is injected subcutaneously into a guinea-pig no immediate effect is produced. The occurrence of a latent or incubative period is a very characteristic phenomenon of bacterial toxin action, and it is always observed however large a dose is injected. If many hundred or thousand multiples of the single lethal dose are injected into an animal the incubation period may be curtailed, but it is not abolished. In the case of diphtheria toxin the latent period may be reduced to 8–12 hours.

Local effect. A soft oedematous swelling is observed at the site of inoculation after about 24 hours. If the dose has been large, especially in the case of certain toxins, this oedema may extend from the pubes to the axilla and clavicle. This swelling becomes firmer during the following days and necrosis of the skin may occur. Alopecia of the affected area may also be present. The time at which necrosis occurs varies greatly according to the particular toxin injected. With certain toxins necrosis takes place when the dose is just below the lethal limit. (For morbid appearances of local lesions of lymphatic glands, etc., see p. 268.)

General Effect.

Temperature. In the first 24 hours there may be a slight rise of temperature which, in the case of a lethal dose, is succeeded by a fall which continues until death occurs, when the temperature may be several degrees below normal.

Effect on the body weight. During the first 24 hours, or incubation period, the normal increase in the weight of the animal may be undisturbed, and the animal's weight may even rise 6 to 10 grammes. When a lethal dose has been injected a marked fall in the weight is observed in the second 24 hours, and continues until death occurs. If the animal survives the injection for five or six days it may recover, feed well, gain in weight, and appear to be perfectly healthy. Notwithstanding these favourable appearances paralysis may subsequently develop.

Paralysis. The paralysis may appear from the fifteenth to the thirtieth day, rarely earlier or later. The hind-limbs are usually first attacked though sometimes the first signs are apparent in the fore-limbs.

When the hind-limbs are first attacked the paralysis may ascend and affect the trunk and fore-limbs. In such cases the animal lies on its side unable to move, but if food is brought within its reach it may be able to eat quite freely as the muscles of mastication may not be affected. Recovery in such cases is by no means infrequent. The paralysis may progress, affecting the movements of the thoracic muscles and diaphragm, and result in respiratory death. Death, however, may be due to heart failure associated with degeneration of the vagus.

In certain cases of sudden death in guinea-pigs, which had apparently recovered from the acute intoxication and shown no signs of paralysis, the writer has found extensive fatty degeneration of the cardiac muscle to be the only marked morbid change.

Experimental diphtheria paralysis occurs most frequently when injections of living bacilli or of toxin-antitoxin mixtures have been employed, but it can occur from toxin alone. Ransom (1900), Madsen and Dreyer (1902), and the writer have all observed paralyses following injections of toxin.

(*b*) *Intra-cerebral Injection.*

The effects of intra-cerebral injections of toxin were first described by Roux and Borrel (1898). These authors found that toxin injected into the brain was more rapidly fatal than when injected subcutaneously, and that smaller doses were required. Intra-cerebral injection of toxin causes paralysis within 12 hours, often followed by death, the usual lesions being observed, fluid in pleural cavity, congestion of suprarenal glands, etc.

In animals which have previously received large doses (40 c.c.) of antitoxin a little toxin given intra-cerebrally causes death. In this case the disease is confined to the nervous system, and it lasts longer. At the autopsy none of the usual morbid changes are present. All the organs, excepting those of the nervous system, are protected by the antitoxin.

The writer has found that, as the result of intra-cerebral injection of diphtheria toxin, typical late diphtheria paralysis, beginning even after an interval of weeks during which the animal seems perfectly well, may occur in addition to the acute effects described by Roux and Borrel. This effect has been obtained with pure toxin alone and toxin-antitoxin mixtures. A toxin which, on subcutaneous injection, killed in a dose of $\frac{1}{300}$ c.c., caused typical diphtheria paralysis when injected intra-cerebrally in a dose of $\frac{1}{2000}$ c.c.

Physiological Effects on the Heart and Blood Circulation.

Heart failure is the ultimate result of diphtheria intoxication, but whether this is due to the direct action of the toxin on the heart, or is secondary to a vaso-motor paralysis is still a matter of dispute.

As the pathological changes have been described in a previous section (Chapter VII) only a brief reference to those changes which have a bearing on the physiological effects will be made here. In acute toxic cases cloudy swelling of the heart muscle is present within 24 hours, and in cases where the intoxication has lasted 36 to 48 hours extensive fatty degeneration of the fibres is already present, the fat globules being easily demonstrated by acetic acid, perosmic acid, or Sudan red. Romberg, quoted by Heinz (1905), and Mollard and Regaud (1897–99) have described minutely the changes observed in dogs as the result of both small and large doses of toxin causing death from the third to the fifteenth day. They concluded that the marked changes indicated that the weakening of the myocardium is one of the causes of death, but in no case did they observe the symptoms of a typical acute myocarditis.

Late in diphtheria intoxication, often shortly before death, the blood pressure falls as was shown by Enriquez and Hallion (1895), and Beck and Slapa (1895). Romberg and Pässler (1899) came to the conclusion that the fall in blood pressure which occurs at a certain stage of the disease is not due to insufficiency of the heart but to paralysis of the vaso-motor centre. They arrived at this conclusion from the observation that stimuli acting directly cr reflexly on the vaso-motor centre of healthy animals, and thus causing a marked rise in the blood pressure, produce little or no effect on the blood pressure of animals suffering from diphtheria intoxication. On the other hand, they found that the heart was able to deal effectively, as evidenced by a rise in the aortic blood pressure, with an increased volume of blood when this was supplied to it by abdominal massage. The former experiment proved inefficiency of the vaso-motor centre, the latter efficiency of the heart. In regard to the latter observation, however, Beck and Slapa found that when the fall in blood pressure was rapid, going on to death, compression of the abdominal aorta did not cause a rise of pressure, proving the severe injury which the heart had suffered. Enriquez and Hallion in their investigations found that there was progressive paralysis of the vaso-motor centre, for stimulation of the crural nerve or asphyxiation failed to produce a response; the blood vessels themselves were not paralysed, for stimulation of the vaso-constrictors of the vessels was followed by the usual contraction. Pressure on the aorta caused only a comparatively slight rise of blood pressure.

Stejskal (1902) considered that the methods employed by Romberg and Pässler were not satisfactory. A proper estimate of the working capacity of the heart can be ascertained by measuring not only the carotid pressure but also the left auricular pressure, and the ratio between these two gives the quotient of the heart's work.

He used very large doses in dogs and got the effects so rapidly that it is questionable if they were produced by diphtheria toxin solely, or by a mixture of substances such as albumoses from the broth. He concludes that vaso-motor paralysis occurs at an early period but that there are stages when the centre is still capable of stimulation and yet the heart itself fails to respond. The effects of diphtheria toxin on the circulatory system then must be due to both vaso-motor paralysis and injury to the heart itself.

Rolly (1899) thinks that the early fall in blood pressure is due to vaso-motor paralysis, but that this is soon followed by paralysis of the heart, which in spite of artificial respiration rapidly causes death. This heart paralysis is not secondary to the insufficient supply of blood, causing loss of excitability, as was suggested by Romberg and Pässler, but is a direct paralysis. Rolly points out that even when vaso-motor paralysis is complete and the blood pressure is quite low, if artificial respiration is maintained, the heart goes on beating for hours. Rolly also by Hering's method of employing a heart-lung circulation in an animal 24 hours after the injection of diphtheria toxin demonstrated beyond doubt, that in experimental diphtheria intoxication neither paralysis of the vaso-motor centre nor stoppage of respiration can alone be regarded as the cause of death, but that independent of these actions, and even when the heart is separated from the vascular system, heart-death occurs. Rolly could not confirm Fennyvessy's (1896) observation that diphtheria toxin acts directly on the isolated frog's heart, nor could he find evidence of direct toxic action on the rabbit's heart isolated by Hering's method. Even when 60 lethal doses were introduced into the small volume of blood in the heart-lung circulation, the heart continued to beat strongly and regularly for two hours. The toxin never produces an immediate toxic effect on the heart, but after a definite latent period a heart paralysis comes on independent of the earlier vaso-motor paralysis. After the latent period has passed, replacement of the toxic blood by normal blood can never save the life of the animal, even when it is introduced before the onset of the heart symptoms. According to Courmont and Doyon's theory of toxin action the latent period is due to the fact that the injected toxin does not itself give rise to the toxic effects, but acting like a ferment causes the formation of the toxic substance which acts directly on the animal body. Rolly however failed, as many others have failed, to find this substance in the blood, for the blood taken from animals far advanced in diphtheria intoxication produced no effect on healthy animals. The toxic effect on the heart, according to Rolly, is due to an absorption of toxin by the heart muscle causing a functional change. He thinks that the sudden onset of the heart symptoms and their rapid development are against the view that they are due to anatomical changes.

According to Heinz (1905) the diphtheria toxin acts injuriously from the outset, for even at the end of the latent period (20 hours) marked cloudy swelling and even fatty degeneration are visible in the muscle fibres. He points out that in considering the influence of the lesions accompanying diphtheria intoxication on the working capacity of the heart, its extraordinary reserve power must be kept in view. When the pathological changes are continuously progressive a stage must come when the heart begins to flag, the beat becomes weak and arhythmic, and the blood pressure sinks. The sudden onset and rapid progress of the heart failure are not, therefore, as was maintained by Rolly, incompatible with the view that the anatomical cardiac lesions are the cause of the heart failure and fall of blood pressure.

THE FATE OF THE TOXIN IN CORPORE.

The fate of diphtheria toxin *in corpore* depends on various factors; the susceptibility or insusceptibility of the injected animal and the manner of introduction of the toxin being among the most important. The subject will be dealt with under the following headings:

1. In susceptible animals.
2. In refractory animals.
3. Behaviour in the alimentary canal.
4. Behaviour when applied to other mucous membranes.

1. *In Susceptible Animals.*

When toxin is injected into the blood stream of susceptible animals it disappears with remarkable rapidity. Bomstein (1898) investigated this point by injecting rabbits intravenously with toxin and estimating the toxicity of the blood per c.c. at the time of introduction of the toxin and at subsequent intervals of time. The fall in the toxicity is shown in the following short table:

	Hours after injection :—	1	3	5	8	10 to 12
Fraction of original toxin injected	**When original toxicity per c.c. = 5 M.L.D.**	·6	·33	·25	·2	·18
	When original toxicity per c.c. = 2 M.L.D.	·5	·25	·18	·15	·12

It will be noted that immediately after the injection the rate of disappearance is very rapid, but that later it becomes more gradual. De Croly (1897) in similar experiments found that the toxicity was unaltered five minutes after the injection, but that in two hours, a half had disappeared. Dönitz (1899), by employing another method, referred to elsewhere, also demonstrated the rapidity with which the toxin disappeared from the blood and became fixed, at first loosely, and ultimately firmly, onto the cells and tissues of the body. He found that, when seven lethal doses of diphtheria toxin were injected intravenously into a rabbit, the quantity of antitoxin which had been sufficient to neutralise the toxin when the two were injected simultaneously, was no longer able to save the life of the animal fifteen minutes after the injection of the toxin. A larger dose of antitoxin could at that period still save the animal, but after an interval of 1½ to 2 hours, no matter how much antitoxin was injected, the animal died. Heymans (1898) injected a minimal lethal dose of tetanus toxin into an animal, and immediately removed the blood which was replaced by transfusion with the blood of a healthy animal. In spite of the brief interval of time between the injection of the toxin and the removal of the blood, enough toxin had become fixed to cause the animal to succumb to typical tetanus.

The rapid disappearance of the toxin from the blood is not due to its excretion by the kidneys or intestines, for Bomstein failed to find it either in the urine or in the contents of the small intestine after the injection of large doses. Similar negative results were obtained by Goldberg (1899) in the case of tetanus toxin. Cobbett (1900) showed that, after large injections, diphtheria toxin can be demonstrated in the urine. This appears to occur only when the system is flooded with the toxin. Salter's (1898) observation that toxin may be excreted with the sweat "lacks confirmation" (Oppenheimer). According to Ehrlich the rapid removal of toxin from the blood is due to the avidity of the receptors of certain cells for the toxin. In the case of diphtheria toxin the receptors are probably widely distributed throughout the body, in the connective tissues and in various organs. Up to the present it has not been demonstrated that the cells of any organ have a peculiar affinity for diphtheria toxin such as the nerve cells have for tetanus toxin.

2. *In Refractory Animals.*

An animal may be refractory either because it has some protective substance to neutralise the toxin, or because it possesses no cells sensitive to the action of the toxin. As reference has already been made to the natural occurrence of antitoxin in man and animals and to the explanation of natural immunity from Ehrlich's standpoint, only a few words are necessary here in regard to these points. Susceptibility, according to Ehrlich, is dependent on the number and avidity of suitable receptors. These are absent or of defective avidity in refractory animals, hence the toxin has no point of attack on the body. From this it also follows that antitoxin should occur only in the blood of those normal animals which are susceptible to the action of toxin, since in these only can superfluous receptors be thrown off into the blood. This is in accordance with observation. So far the existence of normal antitoxin has been demonstrated in susceptible animals only (man and horse). As a general rule only susceptible animals furnish antitoxin in large amount as the result of immunisation. Wassermann and Futaki's observation on the combining power of brain substance with tetanus toxin is of interest in relation to this subject.

It has been shown by a number of observers (Wassermann, Futaki, Metchnikoff, and Asakawa), that the combining power of the emulsified brain of an animal is directly proportional to the *susceptibility* of that animal to tetanus toxin. For example, the brain of the susceptible guinea-pig has a powerful combining action, that of the more refractory domestic fowl has a weak combining action, whereas the brain of the turtle which is altogether refractory has no combining action.

As might have been anticipated from these results the toxin remains circulating unaltered in the blood of such refractory animals for a long period. Metchnikoff found that tetanus toxin injected subcutaneously or intraperitoneally into the marsh turtle (*Emys orbicularis*) passes into the blood and persists there for a long time. In one case the blood of a turtle four months after it had been injected produced tetanus in mice. Notwithstanding the long period of contact with the animal body antitoxin is never produced in these animals. Similar observations were made in certain fishes and in the scorpion. The toxin appears in such cases to be eliminated by a slow process of metabolism. The alligator (*A. mississippiensis*), which is also refractory to tetanus toxin, if kept at 37° C., develops antitoxin very rapidly even within 24 hours, whereas at lower temperatures it gets rid of the toxin but produces no antitoxin. The explanation of the condition in this case is difficult on Ehrlich's hypothesis. In certain cold-blooded animals such as the frog (Morgenroth), and in hibernating animals such as the marmot (Billinger and Meyer), the haptophorous group combines with the receptors at the low temperature, but the toxophorous group comes into action only when the temperature is raised.

3. *The Behaviour of Toxin in the Alimentary Canal.*

Most of the toxins fail to produce toxic effects when introduced into the healthy alimentary canal. In the case of diphtheria toxin this fact was first demonstrated by Roux and Yersin (1889).

The toxins of diphtheria, tetanus, cholera, etc., injected *per os* or *per rectum*, even in enormous quantities amounting to many thousand multiples of the lethal dose, produce no effect on the experimental animals. Snake venom (Weir Mitchell and Thomas Fraser), ricin (Ehrlich), and botulinus toxin (van Ermengem) may be mentioned as exceptions to the general rule, since they act when introduced into the stomach. Gibier (1896) showed that diphtheria and tetanus toxins injected *per rectum* produced no toxic action in the rabbit, dog or guinea-pig and that repeated doses did not give rise to immunity. Cassin and Charrin (1895) state that the internal coat of the intestine exercises a protective function against the action of certain toxins and that mere contact may modify the toxin. Ransom (1898) found in the case of tetanus toxin that guinea-pigs which had received 300,000 lethal doses by the mouth or about half that dose by the rectum suffered no apparent harm, and Nencki, Sieber and Schoumow-Simanowski (1898), working with diphtheria toxin, found that guinea-pigs which received by the stomach 600 lethal doses of the toxin remained quite unaffected. Rabbits which were injected in the same manner with 100 lethal doses suffered from a slight illness and wasting from which they completely recovered; it is suggested that the illness was perhaps due to other causes.

The reason of this harmlessness of the toxin when introduced into the gut has been the subject of much investigation. Ransom, working with the enormous doses of toxin mentioned, came to the conclusion that a large part of the toxin passed through the gut unaltered and was excreted with the faeces. He gave a guinea-pig *per os* a dose of tetanus toxin sufficient to cause the death of 25 million grammes weight of mice and found in the faeces, passed soon after the injection, sufficient toxin to kill 15 million grammes weight of mice. From these and similar results he concluded that about three-fifths of the toxin introduced *per os* escaped unaltered with the faeces. Nencki, Sieber and Schoumow-Simanowski, on the other hand, working with rather smaller doses of tetanus toxin, failed to confirm the observations of Ransom on tetanus, neither could they succeed in demonstrating the presence of diphtheria toxin in the faeces. Similar negative results were obtained by Répin (1895) and Carrière (1899).

Diphtheria and tetanus toxins introduced into the alimentary canal do not excite the formation of antitoxin (Ransom, Carrière). Notwithstanding Ransom's results most observers think that a considerable destruction of toxin takes place in the alimentary canal, and the factors giving rise to this destruction have been the subject of much investigation. Répin (1895) found that some of the digestive fluids possess a marked destructive action on toxins, both *in vitro* and in the intestine. He attributes the failure of toxins to produce their toxic effects partly to their non-dialysable character, for he found that when introduced into a ligatured loop of gut a large proportion was still present after 12 hours. Charrin (1898) observed that diphtheria toxin submitted to the action of pepsin underwent a destructive digestion or attenuation, and that the hydrochloric acid had only a slight influence in this action—the precipitate obtained by the action of the hydrochloric acid had only a feeble toxicity. Nencki found that the sterile filtrates from emulsions of the stomach, and small and large intestines, had a distinct, but by no means constant, destructive action on diphtheria toxin. The most active filtrate was obtained from the small intestine. Saliva, mucus, and filtered extracts of the lungs, liver and brain, and suprarenal bodies were inactive. Pepsin was active, the acid reaction playing little or no part as was shown by neutralisation. Pancreatic juice is the most important and active of the secretions in its action on diphtheria toxin. Charrin and Levaditi (1899) studied the action of the pancreas on diphtheria toxin. The same volume of toxin was injected into pieces of muscle and pancreas of equal weight, and after being kept at 37° C. for 10 hours the toxin was extracted and tested. The extract from the muscle was still toxic for guinea-pigs, whereas that from the pancreas had lost its toxicity. Carrière (1899), using the ferments, obtained results in the case of tetanus which correspond in the main with those of Nencki, the pancreatin being the most powerful agent. Ptyalin, pepsin, and especially bile had a considerable attenuating power; the intestinal epithelium and the intestinal microbes produced very slight attenuation. The leucocytic oxydases caused a notable attenuation. Cano-Brusso (1901) attributes to the mucous membrane of the alimentary canal a destructive action on toxins. He employed tetanus toxin in his experiments, and the results obtained pointed to a relation between the destructive activity of the mucous membrane and the susceptibility of the animal. For example, there was marked destruction in the case of the susceptible rabbit and guinea-pig, whereas in the refractory dog, cat and hen no action could be demonstrated. Baldwin and Levene (1901) found that pepsin, trypsin and papain do not merely neutralise the toxin but actually digest it. From this they argue that the toxin must be of proteid nature, since proteolytic ferments do not affect non-nitrogenous substances.

These results in regard to the behaviour of the toxin in the alimentary canal may be briefly summarised as follows. The absence of action of toxin when introduced in this manner may be due to a number of causes. The fact that toxin does not pass through an unbroken animal membrane has an important bearing on the subject. This alone is not a sufficient explanation, for certain toxins, though incapable of diffusing through animal membranes, are still capable of producing toxic effects from the alimentary canal. A great destruction of toxin must take place in the alimentary canal, for, in the case of moderate and even large doses, it frequently occurs that no toxin can be demonstrated in the faeces. This disappearance is due to a number of factors which are probably of varying importance according to the toxin employed and the conditions of the experiment: 1. the intestinal microbes, 2. the cells, 3. the secretions passing into different parts of the canal. These cause a great destruction of toxin, the bulk of the evidence being in favour of the pancreatic secretion constituting the most powerful destructive agent in the case of diphtheria toxin. The first two are probably the least important.

Action of Toxin on other Mucous Membranes.

To ascertain the action of diphtheria toxin on the mucous membranes of the respiratory tract, Roger and Bayeaux (1897) employed the method of intratracheal injection in rabbits. In some of the animals false membranes were produced on the glottis and trachea giving rise to dyspnoea. From the results obtained by them they concluded that diphtheria toxin is capable of producing false membranes without previous lesion of the mucosa.

Copez (1897) investigated the influence of diphtheria toxin on the conjunctiva. He employed a single instillation and, failing to obtain any pathological effect, concluded that the toxin cannot produce lesions if the corneal epithelium remains intact. Morax and Elmassian (1898) instilled dilute diphtheria toxin (1 in 5) every three minutes for eight to ten hours into the conjunctival sac of rabbits. They obtained all the pathological effects of diphtheria toxin, including the formation of false membrane. Some of the animals died of acute toxaemia, others developed cachexia accompanied by loss of weight.

CHAPTER XVI.

THE FORMATION OF ANTITOXIN IN THE BODY.

Discovery of antitoxins. Active immunisation. Phenomena which occur during immunisation. "Marche ondulatoire." The technique of immunisation in animals (1) with toxin modified by (*a*) chemical agents, (*b*) heat, (*c*) antitoxin (toxone), (2) with toxin only. Discussion of the value of subcutaneous and intramuscular, compared with intravenous, injection of toxin. The period of maximum concentration of antitoxin in the blood. The operation of venesection. Variability in the capacity of horses to produce antitoxin. Subsequent history of diphtheria-antitoxin horses. Active immunisation in man. The effects of various chemical and physical agents on diphtheria antitoxin; Heat, Low temperature, Light, Gases. Filtration. The testing of antitoxin. Ehrlich's method. The French methods. Attempts at the chemical isolation or concentration of antitoxin. The mechanism of antitoxin production. Ehrlich's theory.

The Discovery of Antitoxins.

THE treatment of every disease, to be scientific, must be preceded by a knowledge of its pathology including its causation. The discovery of antitoxin followed a series of events each of which formed a necessary link in the chain. We need only refer to Pasteur's epoch-making fundamental work on the causation of infective diseases which formed the basis of bacteriology and immunity, and Koch's extension of that work by which it became more and more an exact science. From an early period in the history of immunity there has been a tendency for investigation to be divided into two schools, the one dominated by the idea that the body fluids are chiefly concerned in the protection of the organism, the "humoralist's" view; the other, which attributes the most important action to the cells, the "cellular" view, of which the chief supporter is Metchnikoff. More recent work has shown that these views are not necessarily antagonistic as was thought at one time.

The work of Metchnikoff and his school, on which is based the theory of phagocytosis, has done much to make clear the phenomena which take place in the struggle between the invading microbes and the body of the host. According to this view, immunity depends on the activity of the phagocytes, which have the power of moving towards and englobing microorganisms, which they afterwards kill and digest. The phagocytes have a positive chemiotaxis to the substances secreted by the microbes, and can themselves produce substances which are capable of preparing the bacilli for phagocytosis. The cells which have these functions are mesoblastic in origin and include most of the leucocytes (with the exception of the mast cells and lymphocytes), the endothelial cells, connective tissue cells, certain cells in the liver, and so on. The wonderful manner in which the evolution of the process is traced from a simple act of nutrition in the lower forms of animal life to a complex protective mechanism in the higher vertebrates, the ingenuity and wide range of the experiments, and the beautiful simplicity of the conception make Metchnikoff's work one of the most remarkable achievements of biological science. The main theses of the hypothesis are now almost universally accepted.

Another group of workers, devoting their attention mainly to the body fluids, including the blood serum, obtained results which in the course of their development led to a series of discoveries of the highest importance in the field of immunity. Fodor (1887), Nuttall (1888), Nissen (1889), Behring (1888–89), and Buchner (1889, 1890–3), discovered that the blood serum had certain bactericidal properties of a specific character. The bactericidal substance in normal serum was named by Buchner "alexin." It was shown by Nuttall to be thermolabile, being destroyed by an exposure of half an hour to a temperature of 55—60° C. It was soon observed that the serum of naturally immune animals had in some cases a more powerful bactericidal action than the serum of susceptible animals.

The next great step was the observation made by Nissen and Behring that the serum of artificially immunised animals possessed this property in a very marked degree as compared with the serum of normal animals. They immunised guinea-pigs with the *Vibrio Metchnikovi*, and found that the serum of the guinea-pigs so immunised was strongly bactericidal in contrast to the serum of normal guinea-pigs. Bouchard (1890) made the same observation in the case of the *Bacillus pyocyaneus*.

In certain cases of acquired immunity no bactericidal substance could be demonstrated in the blood, and thus attention was directed to a condition the study of which ultimately resulted in Behring's discovery of antitoxic immunity. The discovery and isolation of the *Bacillus diphtheriae,* the specific cause of the disease, by Loeffler (1884, see Chapter I, p. 28) prepared the way for a minute study of the disease, which resulted in a thorough knowledge of the pathogenic processes involved. Roux and Yersin (1888) first succeeded in obtaining toxin from the diphtheria bacillus in broth culture, and Brieger and Fränkel (1890) published confirmatory results.

The demonstration of the possibility of obtaining these toxins in culture media formed a link in the chain of events, the importance of which in relation to immunity was widely recognised, and resulted in a number of workers in Koch's laboratory attacking the problem of immunity to diphtheria. Fränkel (1890) succeeded in obtaining immunity by means of toxins which had been heated to 60° C. and Behring (1890) by means of toxin to which iodine trichloride had been added.

From this brief outline of the history, we can see how the psychological moment had arrived for the discovery of antitoxic immunity. Behring and Kitasato in 1890 published the results of their experiments on diphtheria and tetanus, and in this publication announced the important discovery that an animal, immunised against tetanus or diphtheria, produces in its blood substances which are capable of neutralising the toxic action of the specific poison; that the blood serum, when injected into other animals before the toxin, can prevent the toxic action, and when injected even after the onset of symptoms can save the life of the animal. This substance, "antitoxin," was shown to possess neutralising, preventive and curative properties not only against the toxin but also against the infecting bacillus. The sera of the normal animals which they tested did not possess these properties. Though the results published by Behring and Kitasato concerned both tetanus and diphtheria, Behring (1893) in his history of diphtheria makes it clear that the discovery was first made by himself in the case of diphtheria, and that only after the diphtheria experiments were completed was the experience obtained from them applied by Behring and Kitasato conjointly to the subject of tetanus.

This work of Behring's must be regarded as the foundation of serum therapeutics, though the idea of using the serum of immune animals as a curative agent in other animals had occurred to a number of workers. Among those for whom a claim of priority has been made may be mentioned Héricourt and Richet (1888) who believed that the blood serum of a dog, which had received injections of *Staphylococcus pyosepticus*, protected rabbits against that organism, and Babes and Lapp (1889), who showed that the serum of dogs immunised against rabies contained substances which, when injected into other dogs, were capable of protecting them against rabies. These investigations are chiefly interesting as containing the germ of a great idea which came to fruition in Behring's work.

Behring (1893) gives a list of the methods of immunisation which were employed during this experimental stage of the investigation:

1. The treatment of guinea-pigs with bouillon cultures of the diphtheria bacillus sterilised by heat (C. Fränkel).
2. The treatment of guinea-pigs with broth cultures of the diphtheria bacillus treated with iodine trichloride (Behring).
3. The treatment of guinea-pigs with the body fluids of animals (living or dead) infected with diphtheria (Behring).
4. The cure of guinea-pigs infected with diphtheria by local treatment with different chemical agents (Behring).
5. The preparation of guinea-pigs and rabbits with hydrogen peroxide.
6. The preparation of guinea-pigs by means of a combined method with the object of reaching a high degree of immunity. In this case the animals were first treated with weakened cultures and afterwards with gradually increasing virulent cultures, or with unmodified toxin (Behring and Wernicke).
7. The treatment of rabbits by the subcutaneous injection of a heated precipitate of calcium containing diphtheria toxin (Behring and Wernicke).
8. The treatment of dogs with increasing doses of an unmodified diphtheria toxin and with unmodified broth cultures of *B. diphtheriae* (Wernicke).
9. The feeding of guinea-pigs, rabbits and dogs with diphtheria toxin (Behring and Wernicke).

For the immunisation of larger animals, such as sheep, Behring (1895) stated that he preferred the combined method.

These fundamental results obtained by Behring, Kitasato, C. Fränkel, and Wernicke soon led to the subject being carried beyond the experimental stage. Behring and Boer, Schubert and Canon, and Kossel and Korte tested the serum on children with favourable results. Aronson (1893–4) prepared a serum which gave excellent results when used on patients by Katz and Baginsky. It was, however, only after Roux's remarkable work first published in September 1894 at the International Medical Congress at Budapesth, that the antitoxin treatment of diphtheria became established on a firm practical basis. Roux in his address, and in his work published in collaboration with Martin and Chaillou (1894), gave such a clear exposition of the methods and the results that a very wide interest and enthusiasm were aroused. In this work Roux gave exact details as to the methods of growing the bacillus and obtaining the toxic filtrates. The processes involved in immunising the horses, in obtaining the serum, the result of testing the serum experimentally on small animals, and the influence of the treatment on patients were all stated in the most lucid and precise terms. Indeed, the work of Roux and his colleagues was so complete that it left room for improvement and investigation in little else than detail.

ACTIVE IMMUNISATION.

The active immunisation of animals and man is carried out by the repeated injection into the body of toxin, or of toxin modified by the action of physical or chemical agents or by antitoxin. A preliminary passive immunity may be conferred by the use of antitoxin. In rare cases the unfiltered cultures are employed. A discussion as to the relative value of the different procedures will follow under the heading "Technique of Immunisation" (p. 506).

The Phenomena which occur during Immunisation.

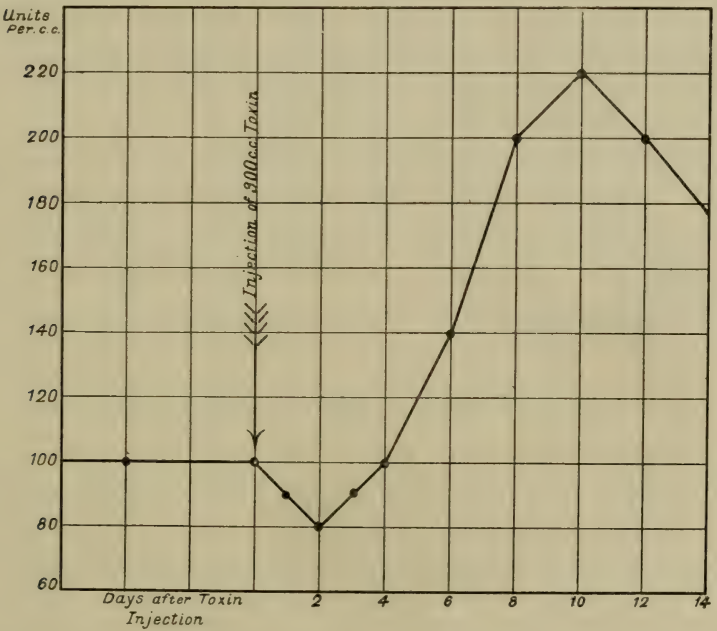
At the beginning of the immunisation the injection of toxin into the horse, goat, etc., is in many cases followed by swelling, oedema, and tenderness at the site of inoculation, with rise of temperature, loss of appetite, and the appearance of general malaise. These phenomena last for a few hours or days, according to the amount of toxin injected and the sensibility of the particular animal to the toxin. Sometimes in the horse, throughout the whole process of immunisation, the effects produced are of the most trivial character, the animal remaining apparently unaffected and gaining weight whilst under treatment.

The study of the rise and fall in the amount of antitoxin in the animal body during immunisation was first undertaken by Brieger and Ehrlich. From the investigation of the milk of a goat immunised against tetanus, they demonstrated how the antitoxin production was influenced by toxin injections in an actively immunised animal. When the antitoxic value was plotted out as a curve its undulations were found to assume a definite form. The relation of the fluctuations in the amount of antitoxin in the blood to the injection of toxin was further studied in other immunisations, for example, by Salomonsen and Madsen in diphtheria, by Morgenroth in rennin, and by Forssman and Lundstrom in botulism.

In all the cases of immunity against toxins, which have hitherto been investigated, these fluctuations have borne a general likeness to one another, the resulting curves being of similar configuration, so that they appear to obey a general law. Salomonsen and Madsen were able to study the "marche ondulatoire" of the immunisation in the case of a milch mare, which had been immunised against diphtheria toxin. They ascertained the antitoxic value of the blood serum and of the milk, and were able to confirm fully the results previously obtained by Ehrlich and Brieger.

Four phases can be distinguished in the fluctuations following a toxin injection (see Chart 1):

Chart 1, *showing the effect of a Single Toxin Injection on the Antitoxin Curve. From the* 10*th day there is a fall at first rapid, later more gradual, the curve ultimately running almost parallel to the base line, as in the position seen to the left before injection.*



First Phase. Immediately after the injection of the toxin there is a fall (or negative phase) in the antitoxic value of both the blood and the milk. This lasts for two or three days and is followed by the second phase.

Second Phase. During the second phase the antitoxic value rises till it attains a higher level than it possessed before the injection of toxin. The maximum of the rise is reached about the tenth day after the injection and is probably of very brief duration.

Third Phase. During the third phase the antitoxic value begins to fall, at first rapidly, later more slowly.

Fourth Phase. During the fourth phase the antitoxic value reaches a fairly constant level or position of equilibrium, during which the curve runs almost parallel to the base line.

Salomonsen and Madsen drew attention to the interesting point, that the fall in the antitoxic value occurring immediately after the injection of the toxin could not be accounted for by the neutralisation of antitoxin by the toxin injected. In one case, for example, the injection of 1 litre of a particular toxin caused a fall in the antitoxic value from 100 to 65 units per c.c. Now 3·8 c.c. of the blood of this horse if mixed *in vitro* with a litre of the toxin would have completely neutralised it. In a later communication Salomonsen and Madsen believed they could demonstrate a similar fall in the antitoxin of the blood by the administration of morphine, and an increase by the administration of pilocarpin; results which supported their views in regard to a negative phase and to the secretion of antitoxin by the cells.

THE TECHNIQUE OF IMMUNISING OF ANIMALS.

1. With toxin modified by (*a*) chemical agents, (*b*) heat, (*c*) antitoxin (toxone).
2. With toxin only.

1. *Immunisation with modified Toxin.*

(*a*) *Chemical agents.* In some laboratories the method of modifying the toxin by iodine trichloride is still used during the early stages of the immunisation.

(*b*) *Heat.* A modification of the method first successfully employed by C. Fränkel was suggested by Cartwright Wood (1896), who grew the bacillus on a mixture of serum and broth, filtered the culture through a Pasteur-Chamberland candle, and heated to 65° C. for one hour. The horses received large doses of this fluid, 380 c.c. in three doses in the first twelve days. It was claimed for this method that it was safe and rapid and that the toxin was essentially different from ordinary toxin. It is probable, however, that we are dealing either with toxoids or with a toxin which has undergone a fractional destruction. After a considerable experience of this method the writer abandoned it for the method described later (p. 509).

(*c*) *Toxin modified by, or employed together with, antitoxin.* After the demonstration of the wonderful preventive power of antitoxin, it naturally occurred to a number of workers that the dangers attending the early stages of immunisation might be averted by the use of antitoxin given before or at the same time as the toxin, or as a toxin-antitoxin mixture.

Nikanorow (1897) found in the case of two goats that, under the same conditions, the one which received toxin on one side of the body and antitoxin on the other side gave an antitoxin value of 45 antitoxin units, whereas the other, which received only toxin, gave 15 antitoxin units. He employed also, in the case of the horse, a number of injections of antitoxin before beginning the treatment with toxin, and claimed that one could thus obtain rapidly and safely a very high antitoxic value.

Kretz (1901) in the course of a study of the paradoxical phenomenon, that an animal may die of toxaemia and yet have a large quantity of antitoxin in its blood, performed several immunisations, which are of interest in relation to the influence which antitoxin injections may exert on the production of antitoxin by the animal body. The results obtained in the case of four horses were as follows:—

Horse	Method of administering toxin and antitoxin	Method of injection	Resulting value of antitoxin
1.	Toxin and antitoxin mixed at room temperature for 18 hours	Intravenous	>2 A.U.[1]
2.	Do. do. do.	Subcutaneous	=3 A.U.
3.	Same quantity of serum. Toxin 12—15 hours later	Intravenous	=c. 20 A.U.
4.	Do. do. do.	Subcutaneous	+100 c. 500 A.U.

It would thus appear that hyper-neutralisation of the toxin is unfavourable to antitoxin production, whereas preventive injection of antitoxin at least allows antitoxin production to proceed. Whether it hinders or aids cannot be ascertained from these experiments. Dzierzgowski (quoted by Eisenberg, 1903) failed to obtain any antitoxin by injecting neutral mixtures of toxin and antitoxin into four goats, two dogs and two horses.

Arloing, Nicolas and Antoine (1901) investigated the antitoxin production in dogs injected with mixtures of antitoxin and toxin, or antitoxin and bacilli. The antitoxic value of the blood serum was so low that it could be altogether accounted for by the antitoxin injected.

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[1] A. U. = antitoxin units (see p. 521).

Arloing and Nicolas (1901) carried these experiments further, using the ass as the experimental animal. In one case, after a long treatment, a very weak antitoxin was obtained by the simultaneous injection of antitoxin and toxin in different parts of the body. In a second experiment they used three animals. The results of these experiments are shown in tabular form below.

Animal	Immunised with	Antitoxic value of the serum per c.c.	Preventive strength
Ass 1	Toxin alone: 131 c.c.	80 A.U.	1 : 50,000
Ass 2	Toxin and antitoxin at different places: 446 c.c. toxin 149 c.c. serum	60 A.U.	1 : 5,000
Ass 3	Mixture of toxin and antitoxin as above	10 A.U.	1 : 500

N.B. The antitoxic value of the serum was ascertained 20 days after the last injection.

It is clear from this table that the best results are gained by the use of toxin alone; where the toxin and antitoxin are injected at separate parts of the body a fairly high antitoxic value is reached; the poorest result is that obtained by the use of a toxin-antitoxin mixture.

Atkinson (1903) gives the results obtained in a series of 100 horses. Over-neutralised toxin-antitoxin mixtures were employed in the first stages, and four months were required to reach a maximum antitoxic value. The doses were repeated every five to seven days, and were increased each time by a quarter to a half. By this method one half of the horses develop sera of 300 units per c.c., and one-fourth develop sera of 500 units per c.c. or more.

Madsen and Dreyer (1901) by immunising with mixtures of toxin and antitoxin in which only the toxone was free succeeded in obtaining antitoxin; an observation of great importance from the theoretical standpoint.

Since the publication of the work of Dzierzgowski and Kretz, Morgenroth has shown that the union between diphtheria toxin and antitoxin is not so rapid as had been imagined, consequently it seems probable to the writer that the slight antitoxin production is due to a small amount of free toxin reaching the necessary cells, before combination has had time to occur. The same argument can be applied to the case where antitoxin is given as a preventive inoculation at the beginning of immunisation as in the work of Kretz and others. If this view be correct it has yet to be proved that the previous injection of antitoxin is of any advantage. The writer, from personal observation, has a strong impression that the injection of antitoxin in the early stages of immunisation is unfavourable to the attainment of a very high antitoxin production. These considerations, and those dealt with in the following section, led the writer to adopt a method of immunising with unmodified toxin alone.

2. *Immunisation by Toxin alone.*

The employment of toxin alone as the immunising agent in the preparation of diphtheria antitoxin has been in wide use since the publication of Behring's and Roux's early results.

The methods just described have been introduced with the view of avoiding the risks attendant on the process of immunisation, especially during the first stages, or in the hope of shortening the period necessary to obtain antitoxins of sufficient strength to be of practical value. So far as the writer's experience goes an immunisation which is both comparatively safe and rapid may be attained by the use of toxin only. Safety may be secured by beginning with very small doses of toxin. The chief difficulty in this respect is the varying susceptibility of individual horses. An interesting example of this occurred in his experience. Three horses each received 1 c.c. of a particular toxin as a first injection. One of the three died within five days with all the signs and symptoms of an acute toxaemia, whereas the other two were so little affected by the first injection that they had already received their second injection before the death of the first horse. After this experience the first dose was reduced to 0·1 c.c. but even this was found to be too high for certain animals, so that now an initial dose of about 0·01 c.c. is employed.

In regard to the *frequency* with which the injections ought to be repeated, reference must be made to the antitoxin curves of Ehrlich and Brieger, and Salomonsen and Madsen (p. 505). From their curves the authors naturally deduced that the most favourable time for repeating the injection of toxin occurs when the curve has reached its maximum point. It would seem at first sight that fresh injections at these times would lead to an almost indefinite rise in the antitoxic strength of the blood. As first pointed out by the writer (Dean, 1899) however, the highest antitoxic values are not reached by the procedure based on this theory. Higher antitoxic values are attained by injecting the animals within the first three days after the previous injection, that is, during the period of fall of the curve or negative phase. It would seem as if this must result in a summation of negative phases ultimately causing toxaemia and death. This does not occur, however, and the opinion expressed in the communication, to which reference has been made, has been confirmed and strengthened by the results obtained with more than fifty horses.

To get a correct idea of the events which probably occur during this method of immunisation it is necessary to form a mental picture of a summation, or super-imposition, of curves. Madsen explains the better results which are obtained by injecting every third day instead of on the day when the antitoxin curve is supposed to reach its greatest height, in the following words:

> "The period of production, on an average nine days, can approximately be divided into three periods: three days of incubation, thereafter three to four days to the maximum of production, and lastly two to three days to the end. Thus, an injection given three days after another will have its maximal production at the time when the effect of the first has ceased. In this way no interruption in the increase of the antibody will take place. If the injection is given on the day of the acme, six days or so will pass before the maximum of production is reached, and during those six days a considerable loss of antibody occurs."

The form of curve obtained during such an immunisation was very thoroughly studied in the case of one horse "B," complete charts of the early stages of the immunisation of which are given (Charts 2 and 3). The antitoxic value of the serum of the horse was tested before immunisation, and immediately before every injection a small sample of blood was taken from the jugular vein and its antitoxic value tested. It would be impossible to study the curves of many animals so minutely as was done in this case, seeing that each point in the curve requires for its determination from four to 10 guinea-pigs. The result is not exceptional as the antitoxin in many of the horses reached a higher value in the same time (see Table, p. 514) than is recorded in the case of this animal[1].

Before a fresh injection the swelling, stiffness, slight loss of appetite and rise of temperature resulting from the previous injection must all have disappeared. In the later stages of immunisation slight swelling at the site of injection may be disregarded.

This method of rapid immunisation does not give a larger number of accidents than any other, and has the advantage of giving a higher

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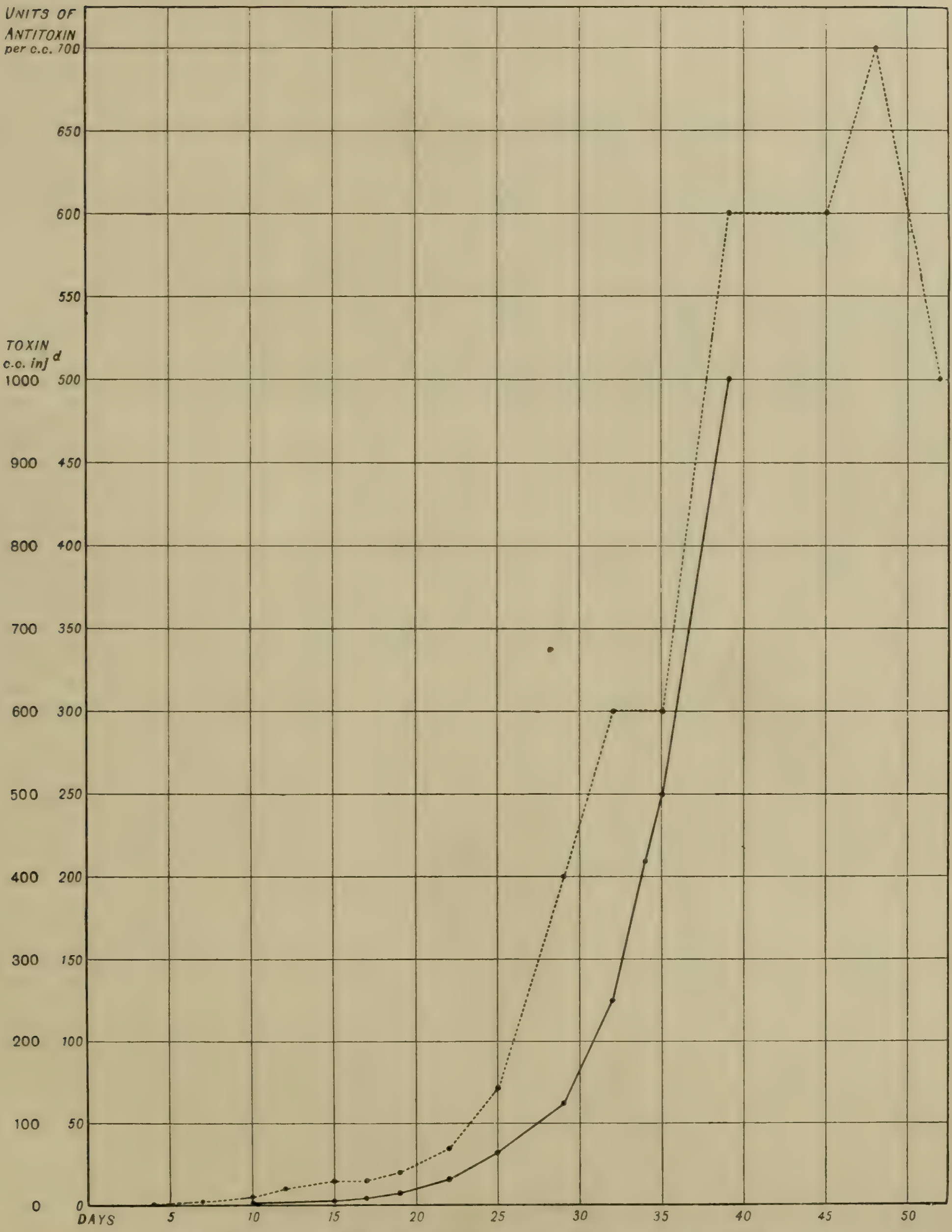
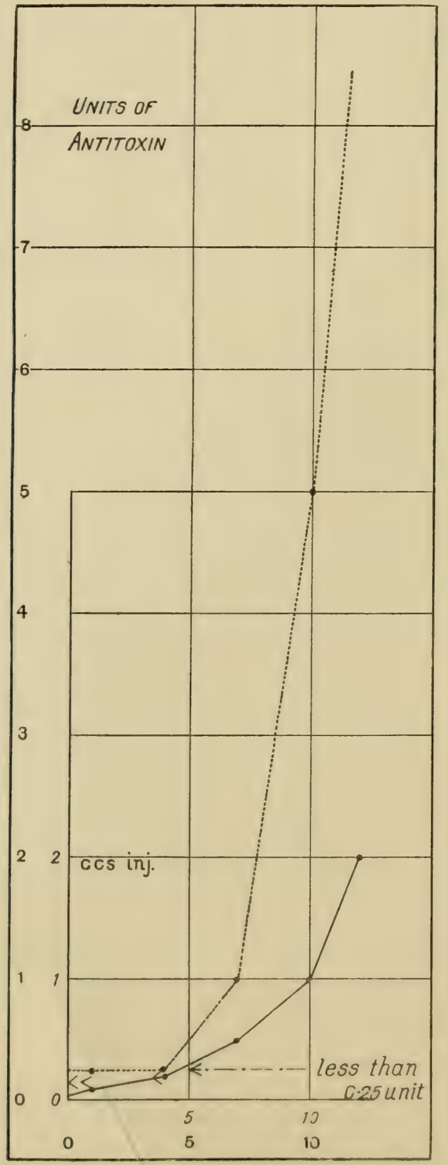
[1] Since this was written Dr T. C. G. Ledingham (1907) has worked out in the Lister Institute and published a similar complete antitoxic curve obtained by the same process of immunisation.

Chart 2. A typical Antitoxic Curve obtained by the rapid method of Immunisation.



The dotted line indicates the number of antitoxic units per c.c., and the uniform line the quantity of toxin injected.

Chart 3, showing the first stages of Chart 2 on a larger scale.



Dotted line indicates antitoxic units per c.c., uniform line volume of toxin in c.c.

antitoxic value in a very short time ; both matters of great practical importance.

The essential points of the method, which the writer has found most useful in immunisation, are :

1. The employment of pure toxin only.
2. The injection during the initial stages of only small doses beginning with say 1/100 c.c., very frequently repeated.
3. The choice of the period of negative phase for the injection, if possible on or before the third day after the previous injection.
4. The increase in the dose over the previous dose must be regulated but is usually 50 to 100 %.
5. By this method when a final injection of say 1,000 c.c. has been reached, the antitoxic value is found in many cases to have attained its maximum. This occurs within a period of seven to eight weeks.

The following Table gives the dates and doses of a typical immunisation.

Type of Immunisation

May 31st	0·01 c.c. Toxin[1]
June 1st	0·02 ,,
,, 3rd	0·1 ,,
,, 4th	0·25 ,,
,, 6th	0·5 ,,
,, 8th	1·0 ,,
,, 10th	2 ,,
,, 13th	4 ,,
,, 15th	8 ,,
June 17th	15 c.c. Toxin
,, 20th	30 ,,
,, 22nd	50 ,,
,, 24th	100 ,,
,, 27th	200 ,,
,, 30th	400 ,,
July 4th	500 ,,
,, 7th	1000 ,,

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[1] This toxin kills guinea-pigs of 250 grammes weight in a dose of 1/400 c.c. The early injections were pushed on rapidly in this case as they gave rise to no local or general reaction.

The results obtained in a continuous series of 57 horses are shown in the following Table. In comparison with other methods the mortality is not excessively high ; three horses out of the 57 having died of acute diphtheria toxaemia, an acute toxaemia mortality of 5·3 %.

Thirty-eight out of 54 survivors (70·4 % or 66·7 % of total number) produced antitoxin having an antitoxic value above 500 antitoxic units at some period of the immunisation.

Table of results obtained by the writer using the rapid method of immunisation in a continuous series of 57 *horses.*

Units of antitoxin	No. of horses	Percentage of horses with units indicated	
		On surviving horses	On total horses
1000	7	13	12·3
900	7	13	12·3
800	2	3·7	3·5
700	1	1·9	1·8
600	10	18·5	17·5
500	11	20·4	19·3
400	9	16·6	15·8
300	5	9·2	8·8
200	1	1·9	1·8
less than 20	1	1·9	1·8

Three animals died of acute diphtheria toxaemia.

DISCUSSION ON THE VALUE OF SUBCUTANEOUS OR INTRAMUSCULAR COMPARED WITH INTRAVENOUS INJECTION OF TOXIN.

Cobbett (1896) from his experience suggested that good results might be obtained by a combined method of subcutaneous and intravenous injection. It seems certain, however, that the method of subcutaneous injection is superior to the intravenous. Dzierzgowski (1902) indeed, from a very wide experience, holds that the injection of toxin into the blood stream does not contribute to antitoxin production. He believes that the toxin injected intravenously is at once neutralised as it would be *in vitro*. In the case of subcutaneous injection only a small part diffuses into the blood, the bulk becomes attached to the cells of the subcutaneous tissue which are then stimulated to antitoxin production.

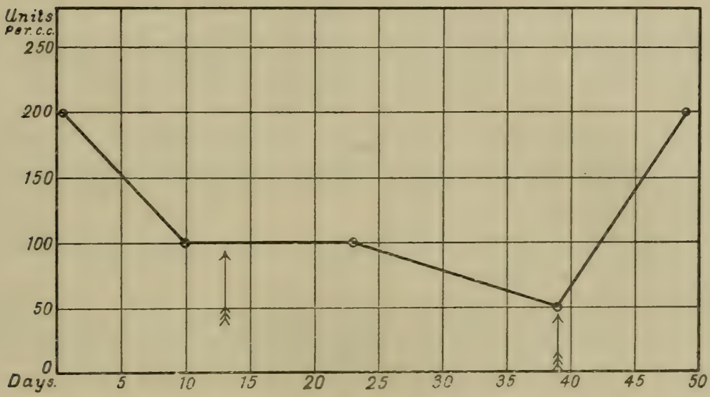
According to this view the production of antitoxin is a process which occurs locally at the site of injection. There is much to be said in favour of this local production of antitoxin. One of the strongest arguments is an experiment by Römer (1901) who showed that if a toxin was repeatedly injected into the conjunctiva of one eye of an animal, that conjunctiva, at a certain stage, could neutralise a given dose of toxin, whereas the other unimmunised conjunctiva possessed no such neutralising properties.

Forssman (1905) investigated the question as to whether antitoxin production resulted from the intravenous injection of botulinus toxin.

He found that intravenous injection did give rise to antitoxin formation but that, whereas the antitoxin curve resulting from subcutaneous injection reached its maximum on the fifteenth day, the curve resulting from intravenous injection was highest on the tenth day. Forssman does not believe that toxin and antitoxin interact in the same way in the animal body as *in vitro.* He shows from Dzierzgowski's own figures that there is evidence of antitoxin production though to a very slight extent.

The writer's experience is limited as to the value of intravenous injection in diphtheria immunisation. In the case of three horses, where a certain rise in the antitoxin curve might have been anticipated as the result of a subcutaneous injection, an intravenous injection of toxin was given. This was followed by little or no rise in the antitoxin curve. The subsequent subcutaneous injection of the same dose of toxin was followed by the expected normal rise. (See Chart 4.)

Chart 4 *of Antitoxin Curve before and after Intravenous and Subcutaneous Injections.*



At the time indicated by the arrow on the left 1000 c.c. of toxin were injected intravenously, and at the time indicated by the arrow on the right 1000 c.c. of the same toxin subcutaneously.

THE PERIOD OF MAXIMUM CONCENTRATION OF ANTITOXIN IN THE BLOOD.

The study of the antitoxin curves (p. 505) has shown that the antitoxin reaches its maximum concentration in the blood about the tenth day after an injection of toxin. In the preparation of antitoxin, therefore, a period of from eight to twelve days is allowed to elapse after the final injection of the series before the removal of the blood.

The Operation of Venesection.

This is carried out under strict asepsis. After sterilisation of the field of operation a small incision is made through the skin over the jugular vein in its long axis, and through this a quill-cut cannula is introduced into the vein which is made to bulge by pressure. To the cannula is attached a rubber tube, by means of which the blood is conducted into sterilised glass vessels. The amount of blood withdrawn varies from 6 to 10 litres according to the size of the animal. The blood may be allowed to clot or may be defibrinated. The serum after being decanted into sterile vessels is the fluid generally known as antitoxin though the actual antitoxin present must constitute only a small fraction of the total serum.

Interval between Venesections.

At least a month is allowed to elapse between the venesections. After a short period of complete rest a few injections of toxin are given to excite the antitoxin production before each withdrawal of blood.

VARIABILITY IN THE CAPACITY OF HORSES TO PRODUCE ANTITOXIN.

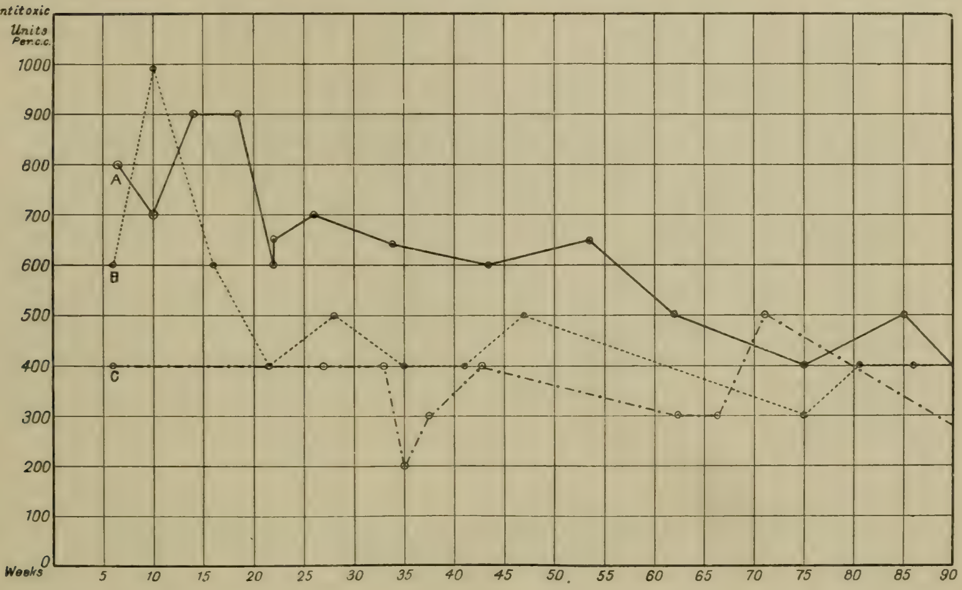
Apart from other circumstances there is no doubt that considerable individual variations occur among horses in regard to their capacity for antitoxin production.

The most striking example in the writer's experience occurred in the case of the last horse in the table on p. 514. This animal was immunised at the same time as another, the same toxin was employed, and the course of immunisation throughout was practically identical in both cases. The serum of the one animal contained 700 A.U. per c.c., whereas that of the other animal had a value of under 20 A.U. per c.c. According to Ehrlich's view this animal must have had some peculiarity or defect in his side-chains. Such a marked absence of the power of antitoxin production appears to be comparatively rare.

SUBSEQUENT HISTORY OF DIPHTHERIA-ANTITOXIN HORSES.

The period during which animals produce a high-grade antitoxin may vary from six months to several years. In the history of most, if not all, antitoxin horses, there comes a time when the injection of toxin is not followed by the usual response of antitoxin production. No matter how severe the stimulation the antitoxin curve goes steadily down. This is shown in Chart 5. If, when signs of this fall begin to appear, a rest of several months is given, the animal may at the end of that time be found to have recovered the property to a large extent.

Chart 5, showing typical Antitoxin Curves of Horses during Diphtheria Immunisation.



A and *B* are common types in the case of the higher grade horses. All the curves, especially *C*, show the tendency to fall after a time in spite of the stimulation of the animals by large injections of toxin.

ACTIVE IMMUNISATION IN MAN.

The active immunisation of man in the case of diphtheria has been little practised. So far as the writer has been able to ascertain only one case has been recorded.

This was carried out in the case of a female patient, one of a group of cases of chronic disease of the upper air passages (Kahnert (1900)). In these cases virulent diphtheria bacilli were found, and the serum of the patient contained antitoxin. As usual in such chronic infections of the nose the injection of antitoxin had no effect on the pathological process. With the view of ascertaining whether an increase of the antitoxin content of the blood would effect an improvement, Kahnert, using a toxin standardised by Ehrlich, proceeded to try active immunisation. The patient received the following injections :—

On 28th March	0·005 c.c.	diphtheria	toxin
5th April	0·01 c.c.	,,	,,
11th April	0·015 c.c.	,,	,,
18th April	0·02 c.c.	,,	,,
30th April	0·02 c.c.	,,	,,
7th May	0·03 c.c.	,,	,,
15th May	0·04 c.c.	,,	,,
25th May	0·06 c.c.	,,	,,
12th June	0·08 c.c.	,,	,,

The injections caused at first painful swellings at the site of inoculation, and had to be discontinued for reasons not connected with the treatment. Whether the great improvement in the condition which occurred was attributable to the treatment could not be decided, as the better seasonal climatic conditions may have had an important influence. The state of the mucous membrane and bacteriological findings were not altered.

Such a heroic measure as active immunisation would be only justifiable in troublesome chronic cases. It is possible that the bactericidal serum suggested by L. Martin may prove useful in cases of this type.

EFFECT OF VARIOUS PHYSICAL AND CHEMICAL AGENTS ON DIPHTHERIA ANTITOXIN.

Effect of Heat on Antitoxin.

Van de Velde (1897) found that temperatures of 60°, 65°, and 70° C. had an injurious effect on both antitoxic and antimicrobial sera. Marenghi (1897) found that serum could be heated at 55° C. in open or closed tubes for five hours and still retain its antitoxic value unaltered.

This is in agreement with Spronck's (1898) view who found that on exposure to 59° C. for twenty minutes only a very slight loss in antitoxic power occurred, whereas the substances which give rise to the unpleasant after-effects, such as skin eruptions and joint-pains, were to a large extent eliminated. Even comparatively low temperatures acting for a long period have a deleterious action. Thus Müller (1898) found that a great loss occurred when the serum was kept at 37° C. for two months.

Dry antitoxin is very resistant to external influences. Camus (1898) found that dry antitoxin resisted a temperature of 140° C. for a quarter of an hour.

Effect of Low Temperatures.

Low temperatures have no bad effects on antitoxin. Bujwid (1897) found that freezing did not lower the antitoxic value even when repeated several times. The temperature of liquid air does not diminish the antitoxic value (Macfadyen).

Bujwid (1897) believed that he could concentrate antitoxin by means of a process of alternate freezing and thawing. He states that during freezing colourless water crystals at first appear, and extend in various directions till most of the serum is frozen, with the exception of a small volume of brownish fluid. The serum, on thawing at room temperature, forms two layers; an upper clearer layer which contains almost no antitoxin, and a lower layer of an intense yellow colour, which contains almost all the antitoxin. The freezing and thawing is repeated two or three times, and in this way a strong concentration of the antitoxin results[1].

Effect of Light on Antitoxin.

Antitoxin is much less sensitive to the action of light than toxin.

Marenghi (1897) found that the Roman July sun acting on antitoxin for several days produced a bleaching of the serum but no effect, or only a very slight one, on the antitoxic value.

Müller (1897) on the other hand found that daylight was injurious. As might have been anticipated red or yellow light was much less deleterious than green or blue light.

Effect of Gases on Antitoxin.

Air, oxygen, nitrogen, hydrogen, and especially carbonic acid were all found by Müller to have an injurious action.

Reference will be made to the action of various ferments in the section on the behaviour of the antitoxin in the alimentary canal.

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[1] The writer failed to find Bujwid's method useful in practice.

Effect of Filtration on Antitoxin.

Martini (1896) first showed that a certain loss in antitoxic value resulted from the filtration of a serum. This was denied by Dzierzgowski (1897) who found that no diminution took place. With the object of solving the question, Cobbett (1898) carried out a number of tests from which he concluded that there is an undoubted loss of antitoxin both with the Berkefeld and Pasteur-Chamberland filters. The loss depends on the size of the pores of the filter, but even in the case of the Berkefeld filter it may reach to 30 % of the antitoxin present, and when Martin's gelatine filter is used all the antitoxin may be kept back (Brodie, 1897). The loss of antitoxin is progressive owing to the pores of the filter becoming more and more blocked. The writer has found that when the ordinary "**V**" Berkefeld filter is employed with a low pressure, the loss is so slight that from the practical point of view it is negligible. In any case the antitoxic value should be determined after and not before filtration.

When kept under moderately favourable conditions, in a dry cool place, and in the dark, diphtheria antitoxin keeps for years, as has been shown by Miller (1905) among others. The higher potency sera deteriorate rather more rapidly than the lower. (For preservation in the dried state *in vacuo* see p. 521.)

THE TESTING OF ANTITOXINS.

Ehrlich's method.

Although it is unnecessary here to give a minute account of all the phases in the evolution of Ehrlich's method of testing antitoxin which led to the method at present practised, a brief description of some of the more important stages is desirable in order to obtain a clear understanding of the basis of the method.

The method first employed by Behring of injecting antitoxin into one part of the animal and living culture into another part was soon abandoned owing to the irregularity of the results. Ehrlich, Kossel and Wassermann (1894) found that greater accuracy was attained by mixing the toxin and the antitoxin *in vitro*, and subsequently injecting the mixture into a guinea-pig, which was early selected as the most suitable animal for test purposes. The standard at this period was an old test toxin of Behring's and the test dose employed was ten times the minimal lethal dose for a guinea-pig of 200 to 300 grammes weight. The unit at that time was the quantity of serum which completely neutralised the action of ten lethal doses of the toxin, *i.e.*, prevented the occurrence of all local or constitutional effects. Later, as the antitoxins became stronger, Behring and Ehrlich, while retaining ten minimal lethal doses as the test dose of toxin, changed the *unit* to ten times the quantity of serum which would completely neutralise the ten lethal doses, that is to say, the antitoxic unit was now fixed as the quantity of antitoxin which would neutralise 100 M. L. D., for a guinea-pig weighing 250 grammes. For example, if 0·1 c.c. of a serum mixed with ten M. L. D. of the test toxin and injected into a guinea-pig behaved towards the animal body as an indifferent substance, then 1 c.c of this serum contained one antitoxic unit. If 0·001 c.c. of a serum produced the same result then the antitoxin contained 100 units per c.c.

Ehrlich at this stage undertook his epoch-making study of the diphtheria toxin, a brief outline of which has already been given (p. 473). He became convinced that both diphtheria toxins and antitoxins in the liquid form were unstable bodies, and found that attempts to use either as standards led to unsatisfactory results. For example, it was found that the neutralisation point of a particular toxin and antitoxin remained constant for more than one year, and yet on closer investigation both toxin and antitoxin were shown to have become weakened. It was observed that in certain cases the loss in antitoxin ran parallel to the loss in toxin, and this fact was especially liable to lead to error. It was therefore clear that constancy of the neutralisation point was no sure guarantee of constancy in the test solutions.

Ehrlich argued that from the chemical side the agents most likely to cause these changes were (1) water, through hydration, (2) oxygen, by causing oxidation, (3) light, and (4) heat. The last two can be easily eliminated, and he proceeds to consider how best to avoid the action of water and oxygen. Though it is possible to obtain diphtheria toxin in the dry state by precipitation with ammonium sulphate there are certain objections, as pointed out by Ehrlich, to the use of such a toxin as a standard. Owing to the presence of a certain amount of crystalline ammonium sulphate in the powder it tends to arrange itself into layers, the upper layers being more toxic than those below. Besides this, a great part of the toxin may be transformed during the process of precipitation into modified substances which are capable of interfering with the testing. As the more suitable substance to work on in the search for a standard, he therefore chose dried diphtheria antitoxin. Dried diphtheria antitoxin is put into a small glass apparatus consisting of two tubes with a connecting tube forming an "H" shaped arrangement. The antitoxin is put into one tube, and phosphoric anhydride into the other to act as a dehydrating agent. The tube containing the dried serum is sealed off and then as complete a vacuum as possible is obtained by using a mercury pump. The second tube is then sealed off and by these means the antitoxin becomes thoroughly dry in what is practically an oxygen-free atmosphere. The tubes are kept in a cool dark place. A number of tubes were prepared in this way, each tube containing two grammes of a dry serum which by the older method had been estimated to contain 1,700 immunity or antitoxic units. Every two or three months one of these tubes is carefully opened and the contents dissolved in 200 c.c. of a 10% salt solution which contains 50 to 80% of glycerine. In this way a test serum containing 17 units per c.c. is obtained. This method of preparing and conserving a standard antitoxin has given uniform and reliable results, and both for practical and theoretical purposes has proved most satisfactory. The unit derived from the older method of testing is a perfectly arbitrary one, and if serum is to be measured by this method the standard unit must first be obtained from Ehrlich. As already indicated the quantity of serum which neutralised 100 M.L.D. of a particular diphtheria toxin which was in use at that time was chosen as the antitoxic unit. Neutralisation was indicated by a complete absence of reaction, local or general, in the guinea-pig. Madsen (1902) has stated that by the physico-chemical methods employed by himself and Arrhenius it would be possible, if the standard unit were altogether lost, to recover it by calculation.

In the older methods referred to the strength of the antitoxin was determined by ascertaining the amount of antitoxin necessary to completely neutralise a certain fixed quantity of toxin. The test was a subjective one and the results were influenced largely by the personal factor. The determination of the presence or absence of slight traces of oedema was a constant difficulty. To abolish this source of error Ehrlich introduced an objective test, namely death, as the criterion in determining the strengths of antitoxins. The test dose of toxin, instead of being the L_0, was now made the L_+ dose. The latter, it will be remembered, is the amount of toxin which, mixed with one unit of antitoxin, causes the death of a guinea-pig of 250—280 grammes weight in four or five days.

The Selection of a Suitable Test Toxin.

The toxin to be employed for test purposes must have reached a condition of stability. It should be stored in a cool, dark place in closely stoppered bottles of large capacity (2 litres), under a layer of toluol. In many cases a period of nine months to one year must elapse after filtration before equilibrium is established. The M.L.D., L_0 and L_+ values are then determined. It is not desirable to use for test purposes a toxin whose L_+ is over 1 c.c., nor should the difference between the L_+ and the L_0 be more than 15 M.L.D. Assuming that the toxin has been previously found suitable in regard to these points it may be used as a test toxin. Its sterility and stability must be ascertained by frequent tests. It may be desirable here to outline briefly the technique of testing.

TECHNIQUE OF TESTING.

The glass vessels, syringes, etc., employed in the process of testing should be sterilised.

The Determination of the L_+ Dose.

The determination of the L_+ dose must be done with great accuracy, and may require several series of tests. The appropriate dilution of Ehrlich's standard antitoxin solution is made containing 10 antitoxic units per c.c. One c.c. of the standard solution is pipetted from the bottle by means of a 1 c.c. glycerine pipette, and put into a flask of 50 to 100 c.c. capacity. Nine c.c. of sterilised tap or distilled water, which may be most conveniently measured by a standard burette, are introduced into the flask. The antitoxin and water are well mixed by shaking. One c.c. of this solution contains one antitoxic unit. A number of small conical test glasses, corresponding to the number of tests to be made, are arranged in a suitable stand. Into each of these test glasses 1 c.c. of the dilution of antitoxin, containing one antitoxic unit, is introduced. From a rough test previously carried out two limits, between which the L_+ dose must lie, have been ascertained. Between these two limits a series of quantities of toxin are fixed on for testing, the spacing between the doses being made as nearly uniform as possible. Each quantity of toxin, beginning with the smallest in the series, is placed in one of the test glasses containing an antitoxic unit. Each mixture of toxin and antitoxin is brought up to a volume of 4 c.c. and after standing

at room temperature for about 20 to 30 minutes is injected subcutaneously into the abdominal wall of a guinea-pig weighing 250—280 grammes. The weight and the condition of the site of inoculation are ascertained and recorded day by day, and eventually the date of the death of the guinea-pig is noted. The mixture which gives rise to the death of the guinea-pig on the fourth or fifth day after inoculation contains the correct L_+ dose of toxin. If two mixtures cause death on this day the smaller of the two doses is the L_+ dose. An example of such a test may be given:—

Test to determine the L_+ dose of Test Toxin No. 30.

Guinea-pig	Mixture injected	Result
1	1 antitoxic unit + ·34 c.c. toxin	Slight local oedema, late paralysis.
2	,, + ·35 ,,	Large local oedema; great loss in weight, death on 6th day.
3	,, + ·36 ,,	Large local oedema, death on 4th day.
4	,, + ·37 ,,	,, ,, ,, ,, 3rd day.
5	,, + ·38 ,,	,, ,, ,, ,, 3rd day.
6	,, + ·39 ,,	,, ,, ,, death in 48 hours.
7	,, + ·4 ,,	,, ,, ,, ,, 48 hours.

From this test it can be seen that the correct test dose (L_+) of this series is ·36 c.c. Previous tests gave the M.L.D. as ·005 c.c., the L_0 as ·31.

$$L_+ - L_0 = \cdot 05 \text{ c.c.} = 10 \text{ M.L.D.} < 15 \text{ M.L.D.}$$

The toxin, therefore, is suitable for test purposes.

The L_+ may now be employed for testing the antitoxic value of any serum by substituting the serum to be tested for the antitoxic unit. In this test the constant is the toxin, the variable the antitoxin. A simple example may be given of the method of ascertaining whether a serum contains one, five or ten antitoxic units per c.c.

Test of Antitoxic Serum for 1, 5 *or* 10 *antitoxic units per c.c.*

Guinea-pig	Mixture injected	Result
8	1 c.c. of the serum + ·36 c.c. toxin	Lives; no oedema.
9	1/5 ,, ,, + ·36 ,,	Large oedema; loss of weight; dies on the 4th day.
10	1/10 ,, ,, + ·36 ,,	Large oedema; dies on the 2nd day.

The serum therefore contains 5 but not 10 units per c.c.

Test of Antitoxic Serum to ascertain whether it contains 400, 450, 500, 550 *or* 600 *antitoxic units.*

Guinea-pig	Mixture injected	Result
11	1/400 c.c. serum + ·36 c.c. toxin	Slight oedema; no loss of weight, lives.
12	1/450 ,, ,, + ·36 ,,	,, ,, ,, ,,
13	1/500 ,, ,, + ·36 ,,	Medium oedema; small loss of weight, lives.
14	1/550 ,, ,, + ·36 ,,	Large oedema; great loss of weight, dies on the 4th day.
15	1/600 ,, ,, + ·36 ,,	Large oedema; dies on the 3rd day.

This serum contains, therefore, 550 but not 600 units per c.c.

The French Methods of Standardising Sera.

The French still employ the method of testing the preventive and curative strength of a serum apart from its toxin neutralising power.

The *preventive strength* is said to be 50,000 when 0·01 c.c. of the serum protects a guinea-pig of 500 grammes weight against a dose of diphtheria toxin which kills a control animal of the same weight in 36—40 hours. The relation between the weight of the animal 500 and the quantity of serum employed is the measure of the preventive strength of the serum. The serum is injected 12 hours before the toxin. Every guinea-pig which has not lost weight after four to six days is considered protected.

The *curative strength* of a serum is estimated in a similar manner. In this case the toxin is injected six hours before the serum. The guinea-pigs surviving on the sixth day are considered cured. In place of the toxin the living bacilli may be employed for the tests.

While fully appreciating the value of Ehrlich's researches into the constitution of toxin, Roux (1900) indicates certain difficulties in accepting all his views. He points out the impracticability of determining the L_0 limit of a toxin, and shows that a mixture, which is apparently completely neutralised for guinea-pigs under ordinary conditions of temperature, is still lethal for the same animals kept in a moist, cold temperature about 2·5° C. Similarly, mixtures are still toxic for small birds though they contain more antitoxin than is necessary for complete neutralisation in guinea-pigs. It may be asked indeed if such a thing as a neutral mixture really exists. He notes also that toxic mixtures when injected before a lethal dose appear to delay the lethal period.

These observations of Roux are of great interest when regarded in relation to the recent work of Madsen and Arrhenius. A comparison of the French method and Ehrlich's method shows, according to Roux, that the latter does not give a complete, or quite accurate, index of the value of a serum.

Two sera were tested by Ehrlich's method and were found to contain, the one, "A," 700, and the other, "B," 200 antitoxin units. M. Momont found that the preventive strength of "A" was 150,000 and of "B" 200,000.

M. Danysz on testing the curative strength found that 10 units of serum "A" produced the same effect as two units of serum "B." The serum therefore, which reckoned by Ehrlich's antitoxic units was three and a half times as strong as the other, was inferior both in preventive and curative properties.

As the result of Roux's statement Marx (1901), working in Ehrlich's laboratory, instituted a series of tests to ascertain whether anti-diphtheritic sera of the most varied origin could possess antitoxic, preventive, and curative properties in varying degrees, or whether the number of antitoxic units was an index of all these properties.

He used the following antitoxins :—Ehrlich's standard dried serum, 1700 A. U., Höchst 1150, Höchst 750, Paris 175 and cow serum 25, and tested their preventive and curative action in relation to their antitoxic strength on rabbits and guinea-pigs.

He came to the conclusion that the toxin neutralising strength (the antitoxic value) of an anti-diphtheritic serum, the immunising and the curative actions are three factors which stand in the closest relation to one another, and that the immunising and curative effects of a serum are directly proportional to the number of antitoxin units contained in it. As Ehrlich's method of estimation is far the easiest and most accurate it is to be preferred to all others.

Madsen (1897), who made a comparative study of the French method and of Ehrlich's earlier method, came to the conclusion that the latter gave the more accurate results. It must be remarked, however, that in this study Madsen was not considering the possibility of sera having different properties in different relative proportions.

The author carried out a series of experiments with the view of ascertaining whether the antitoxic content of the sera of horses at different stages of their immunisation gave an accurate measure of the preventive and curative properties as tested by the French method. The sera selected for the tests were from three horses, one of which, newly immunised, was at the height of its antitoxin production, and had 1000 A.U. per c.c. in its serum ; the second horse was at the stage when the antitoxin curve, in spite of large injections, was steadily falling, the serum containing 300 A.U. per c.c.; the third horse had received no toxin injections for several months; its serum contained 100 A.U. per c.c.

He concluded that the estimation of the antitoxin content by Ehrlich's method gave in the case of these three sera a correct measure of all three factors, the neutralising, the preventive, and the curative. His conclusions, therefore, agreed with those arrived at by Marx.

Cruveilhier (1905) has recently repeated the experiments on which Roux based his opinions, and has fully confirmed them. He employed for his tests living cultures of various origin, and tested sera of known antitoxic strength both for their preventive and curative properties. He concluded that these tests were of great value in estimating the therapeutic value of a serum, and that the usual test for antitoxic strength does not give a correct indication of the efficiency of a serum.

Attempts to chemically isolate or concentrate antitoxin.

Brieger and Ehrlich (1893) attempted to concentrate the antitoxin from the milk of immune animals. They tested a large number of precipitating agents, including acetic acid, tannin, oxalic acid, tartaric acid, acetic acid and sodium chloride, acetic acid and ferrocyanide of potassium, alcohol, metal salts, sodium chloride, phosphate of calcium, magnesium hydrate, and aluminium hydrate. Ammonium and magnesium sulphate, etc. were found to be the most suitable. They used the principle of fractional precipitation and found that the addition of 27 °/₀ to 30 °/₀ of ammonium sulphate gave a precipitate which contained the bulk of the antitoxin, only a trace remaining in the filtrate. In this way they were able to obtain a substance in the form of a powder which was 400 to 600 times as strong in antitoxic power as the original milk.

Brieger and Cohn (1893), employing the milk of animals immunised against tetanus, first clarified the whey by the use of chloroform. They first produced a precipitate with ammonium sulphate, then re-dissolved the precipitate and added basic acetate of lead which throws down a considerable amount of proteid but not the antitoxin. The precipitate resulting from the lead acetate was washed several times in weak alkaline solution, and the filtrates and washings thus obtained were twice treated with ammonium sulphate and the precipitate dried *in vacuo* on clay. The powder was freed from salts by being placed in pure chloroform which allowed the heavier salts to fall and the lighter powder containing the antitoxin to rise to the surface. This concentrated the antitoxin up to 300 or 400 times its original strength. A further purification was attempted by the use of metal salts, alcohol, etc., but without success. On precipitation of the preparation obtained by the use of lead, first with sodium chloride, then with phosphate of soda, and lastly, with ammonium sulphate, the precipitate with the phosphate of soda contained the most antitoxin. It then reached 600 times its original strength.

Wassermann (1894) has improved on the method of concentrating antitoxin from whey. He adds 33 % ammonium sulphate to the whey, filters, and puts the precipitate with a platinum spatula on pieces of clay to absorb moisture. While still damp the precipitate is re-dissolved in one-tenth of the original quantity of fluid. The loss of material is trifling, and practically all the antitoxin is present in the precipitate.

Smirnow (1895) found an antitoxic substance in the magnesium sulphate precipitate from normal, as well as from antitoxic, serum.

Brieger and Boer (1896) succeeded in partly separating diphtheria antitoxin from the adherent albuminous substances by precipitation with neutral salts of the heavy metals. To a fivefold dilution of the diphtheria or tetanus antitoxin double its original volume of a 1 % solution of zinc sulphate or zinc chloride is added. The resulting antitoxin-zinc precipitate is washed with a little water, and dissolved in weak alkaline water (1 : 400 normal alkali). The zinc is precipitated from the solution by the passage of CO_2. If zinc sulphate is used the precipitate contains the antitoxin, but if zinc chloride is used the antitoxin is found in the filtrate. The part containing the antitoxin is dried in the exsiccator to make the zinc-albuminate insoluble, and then the soluble zinc-antitoxin is again dissolved in weak alkali and the remaining zinc again precipitated by CO_2. Brieger and Boer obtained from 10 c.c. of antitoxic serum a deposit of 0·1 gramme which contained all the antitoxin together with albumin, sugar and zinc.

Brodie (1897) precipitated the globulin in four parts by the gradual addition of ammonium sulphate until half saturation was reached. It was found that each of the four precipitates contained antitoxin in almost equal bulk, so that the method did not offer any promise of a possibility of obtaining a stronger solution than could be obtained by re-dissolving the precipitated globulins as a whole. The fact that each of the four fractions contained approximately equal quantities of antitoxin seemed to prove that the globulin precipitate did not carry down the antitoxin by a simple mechanical process. Brodie also stated that antitoxin did not pass through a gelatine filter. These facts appear to indicate that antitoxin is a proteid, belonging, as shown by its solubilities, to the globulin class.

Dieudonné (1897) during a revision of Smirnow's (1895) work failed to find antitoxin amongst the globulins when the precipitation was carried out with carbonic acid. He was able to explain the apparent discrepancy between his results and those of Smirnow by the use of magnesium sulphate, for the globulin thus obtained possessed toxin-neutralising properties. The references already made to the occurrence of antitoxin in the serum of the normal horse (p. 454) make it evident how Smirnow obtained a toxin-neutralising action with the globulin from normal horse serum.

Belfanti and Carbone (1898) could obtain no antitoxin in the precipitate resulting from the use of CO_2 or acetic acid ; but succeeded when ammonium sulphate or magnesium sulphate was used.

Szontagh and Wellmann (1898) made a comparison between normal horse serum and antitoxic serum. The specific gravity and the globulin content were the same in both, and in neither was nucleo-albumin present. The antitoxin contained 0·25 % more of total proteids than the normal serum, a fact to which they attached no particular significance. In both cases the chlorine and ash were the same

They made the important observation that in the antitoxic serum there is a lowering of the freezing point and of the electrical conductivity, which appear to be proportional to the amount of antitoxin present.

Freund and Sternberg (1899) tested the action of various chemical agents on antitoxic serum with the view of freeing the antitoxin from the albuminous substances. They obtained the best results by a continued method of precipitation and salting out. They first added potash-alum solution, dialysed the filtrate, removed the resulting slight precipitate by filtration, and by this means got rid of the albumin. The fluid thus obtained was saturated to a half with ammonium sulphate, and the precipitate, which resulted, after being dissolved and dialysed, was concentrated *in vacuo*.

Marcus (1899) showed that there were two forms of globulin in normal serum; the one insoluble, precipitated by carbonic acid and acetic acid, and by dilution with water and dialysis; the other soluble, precipitable by ammonium and magnesium sulphate.

This observation by Marcus proved of great value to Seng (1899) who examined the qualitative and quantitative relations of the proteids in diphtheria antitoxin compared with those in normal horse serum. Seng found that the method first employed by Sternberg of getting rid of a large part of the proteid matter by a potash-alum solution (one-third volume of 5 % solution) was very helpful. This method, which originated from Freund's experiment, was the first to be described by means of which albumin could be got out of an alkaline solution, the globulin remaining in solution. He found that the chief difference between the soluble globulin from normal serum and from antitoxin was that in the case of normal serum there were present precipitation points at 65° C., 68° C., and 71° C.; at 71° C. all the globulin with the exception of traces was coagulated; whereas, in the case of antitoxic serum flocculi first appeared at 71° C., and 75° C. was reached before the bulk was coagulated. Even after this the traces remaining in solution were much greater. From the qualitative examinations no distinct differences could be made out between the soluble globulin of the normal serum and of the antitoxic serum, and the hopes that conclusions might be drawn from the alteration of the quantitative relation of one body in favour of another were not fulfilled. Yet by the exclusion of the albumin and of the insoluble globulin the field for future investigation was narrowed, since it will naturally be limited to the soluble globulin. Whether the differences in the coagulation temperature and in the optical rotation are due to a change in the globulin or to its combination with an antitoxin of unknown chemical composition is a question which must be left open. This work, and the work of Marcus, tend to show that the antitoxins are attached to the globulins which do not go out of solution by dialysis.

Seng's results throw a clearer light on the results previously obtained by Belfanti and Carbone, and Dieudonné. These soluble globulins correspond to the pseudoglobulin of Pick, to which reference will be made later.

Astros and Rietsch (1900) dilute the antitoxic serum fivefold and add enough sodium and potassium chloride to the diluted serum to bring the concentration of these salts up to 20 %. The mixture, after the addition of 0·5 % phenol, is kept at 33° C. for 24 hours. The antitoxin is thus thrown out of solution together with the globulins.

Atkinson (1899—1900) investigated the fractional precipitation of the globulin and albumin of normal horse serum and of diphtheria antitoxic serum, and tested the antitoxic strength of the various precipitates. He found that the globulins from both normal and anti-diphtheritic sera react in the same way towards reagents. All the antitoxic power of normal and immune serum is carried by the globulin and its fractional precipitates, a certain loss of globulin and antitoxic power occurring during the manipulations. He believes, from the reactions obtained and from the fact that diphtheria antitoxin is contained in normal horse serum and that this natural antitoxin separates with the globulin, that the diphtheria antitoxin is probably a kind of globulin.

Hiss and Atkinson (1900—1901) continued these studies. By the use of magnesium sulphate they obtained a precipitate of globulin which appeared to contain all the antitoxin in the serum, for the precipitate from 1 c.c. of serum protected guinea-pigs against the same amount of toxin as 1 c.c. of the serum. They made the interesting observation that the globulin precipitate is increased as the result of immunisation, for they found that antitoxic sera on the average gave a higher globulin precipitate than did normal sera, and that the serum of the same horse after immunisation gave a larger precipitate of globulin than before immunisation. They conclude that antitoxin formation is probably accompanied by new globulin formation.

Pick (1902) found that on the addition of 21·5 % of ammonium sulphate to anti-diphtheritic serum a precipitate formed, but no antitoxin was present in it = Fraction I.

When the ammonium sulphate reached a concentration of 25·6 % a second precipitation of globulin occurred which still contained no antitoxin = Fraction II.

A fraction of proteid precipitated by the addition of ammonium sulphate up to 38 % contained practically the whole of the antitoxin = Fraction III.

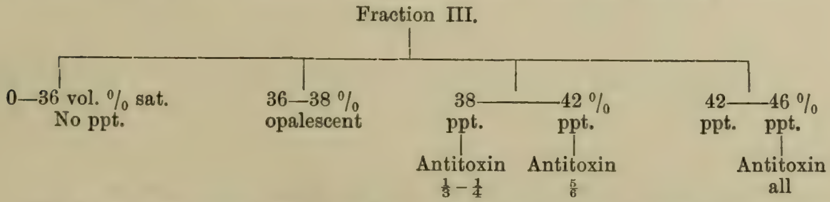
In solution there remained only the serum albumin = Fraction IV.

Pick states that Ide and Lemaire (1899) obtained similar results.

The relations of these fractions to the substances called by Hofmeister "Euglobulin" and "Pseudo-globulin" and their fractional precipitation points with ammonium sulphate, as found by Fuld and Spiro (1900), are shown in the following Table:—

Fibrinoglobulin	Euglobulin	Pseudo-globulin	Albumin
Fuld and Spiro.			
0—21·5 vol. % sat.	28—36 vol. % sat.	36—44 vol. % sat.	100 vol. %
Pick. Fraction I.	Fraction II.	Fraction III.	Fraction IV.
0—21·5 % $AmSO_4$	21·5—25·6 %	25·6—38 %	38—76 %
$0-\frac{1}{3{\cdot}5}$ sat.	$\frac{1}{3{\cdot}5}-\frac{1}{3}$ sat.	$\frac{1}{3}-\frac{1}{2}$ sat.	$\frac{1}{2}$ to sat.
0—28·3 vol. % sat.	28·3—33 vol. % sat.	33·3—50 % sat.	50—100 vol. % sat.

Fraction III when re-dissolved and re-precipitated gave the following result.



The filtrate containing only the albumin had no antitoxic value. The above result was obtained with horse antitoxin. The interesting observation was made that in the case of goat's serum the antitoxin came down, not with the fraction corresponding to the pseudo-globulin, but with the fraction corresponding to the euglobulin. It will be remembered that by Brieger and Ehrlich's method of precipitating antitoxin from the whey of the goat a great loss of antitoxin was encountered during the dialysis. The loss can be explained in the light of the interesting observation made by Pick, that the antitoxin in the case of the goat goes down with the euglobulin, for we know that the euglobulin in salt solution goes out of solution on dialysis.

Pröscher (1902) claims to have obtained by trypsin digestion an antitoxin which gives no proteid reactions. This observation, if confirmed, would be of the greatest interest and importance on both theoretical and practical grounds.

Gibson (1905—1906) precipitates the serum with an equal volume of saturated ammonium sulphate solution, filters, and extracts the residue with a saturated solution of sodium chloride. The globulin containing the antitoxin, the pseudo-globulin, is soluble in the sodium chloride solution, whereas the globulin not containing antitoxin, the euglobulin, the proteids, etc., are precipitated and allowed to sediment. After filtration the sodium chloride solution of antitoxin is precipitated by the addition of a half volume of saturated ammonium sulphate or with acetic acid. The filtered precipitate is pressed dry with paper and dialysed in parchment. A double filtration through Berkefeld filters is employed for sterilisation. One quarter to one half per cent. sodium chloride and a preservative are added. After being tested for potency, sterility, etc. this antitoxin is employed in practice.

By this method a serum, satisfactory in regard to appearance, absence of high viscosity, etc., and of at least twice the original antitoxic potency may be comparatively easily secured.

In a horse which yielded high-grade antitoxin Ledingham (1907) showed that the total globulin progressively increased during immunisation to double its initial value. In a refractory horse which failed to yield high-grade antitoxin no such progressive rise was observed.

Pick's statement, that the antitoxin in the case of the goat is precipitated with the euglobulin and not with the pseudo-globulin as in the horse, has not been confirmed either by Gibson and Collins (1907) or by Ledingham (1907).

Banzhaf and Gibson (1907) found that the serum globulins soluble in saturated sodium chloride solution of the higher fractions are uniformly much more potent in antitoxin per gram of proteid, than are those precipitated by lower concentrations of ammonium sulphate.

THE MECHANISM OF ANTITOXIN PRODUCTION.

Antitoxin was at first regarded as a direct derivative of toxin which by some process taking place in the animal body became converted into antitoxin, in other words, that the antitoxin was a modified toxin. This hypothesis, which was suggested by Buchner (1893), seemed to be a natural deduction from the facts as known at the time. An animal injected with a toxin developed antitoxin in its blood; it seemed, therefore, probable that the animal body had acted on and modified the toxin in such a manner as to convert it into antitoxin. A more intimate investigation of the question raised many difficulties in the way of accepting this view. Exact determinations showed an absence of quantitative relation between the amount of toxin injected and the amount of antitoxin produced in the animal body. Knorr (1899) showed that the injection into an animal of an amount of toxin which could be neutralised by one unit was followed by the production of 100,000 units. Ritchie (1901) in immunising guinea-pigs with tetanus toxin found that twice the amount of toxin gave a serum one hundred times stronger. It has been suggested that the toxin molecule might split up into molecules of smaller size, each containing a group capable of neutralising a toxin molecule. We have reason to believe however, from the work of Brodie, Martin and Cherry, etc., that the antitoxin molecule is probably larger than the toxin molecule, consequently it is difficult to understand how the smaller toxin molecule could be so modified in the animal body as to give rise to a much greater number of larger antitoxin molecules. There is also no evidence in favour of the splitting up of the toxin molecule into parts which go towards the constitution of the antitoxin molecule. A very strong argument against the view that the toxin is directly employed in the building up of the antitoxin was furnished by Roux and Vaillard (1893), who found that in the case of a rabbit immunised against tetanus quantities of blood equal to the total volume contained in the animal's body could be removed within a few days, and yet no diminution in the antitoxic value of the blood could be determined. Similar results were obtained by Salomonsen and Madsen and by the author in the case of goats and horses immunised against diphtheria. The loss of antitoxin which occurs immediately after the removal of blood is quickly made good by a supply which must be derived from the cells and tissues of the body. As this occurs without the re-injection of toxin it is improbable that the latter can in any way be concerned in building up the new antitoxin. These considerations lend increasing support to the view that the animal body furnishes the antitoxin by a process analogous to secretion.

Ehrlich's Theory of the Mechanism of Antitoxin Production.

Ehrlich holds that the fundamental processes of antitoxin production reside in the cells for which the toxin has a selective affinity. The cells may be those of any organ or tissue, but it is essential that they should be capable of being affected by the toxin. The cell protoplasm, which must be regarded entirely from the chemical standpoint, consists of living molecules of great complexity. These molecules have a central nucleus with lateral or side-chains which are capable of binding the molecule to other molecules having the proper affinities. Each molecule of protoplasm has many different kinds of side-chains which have affinities for different substances (see Fig. 3). These side-chains are the means by which the nutrition of the cell is maintained, their normal function being to attach to themselves certain food stuffs. These food stuffs must have their atom groups so arranged that they fit the

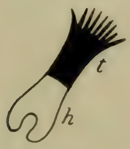


Fig. 1.

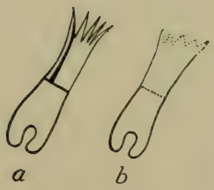


Fig. 2.

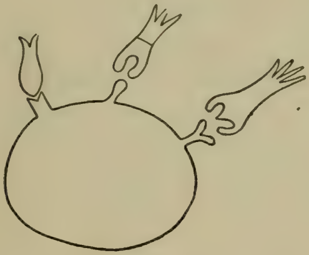


Fig. 3.

Fig. 1. Toxin molecule. h = haptophorous group. t = toxophorous group.

Fig. 2. Toxoid molecule with (*a*) a trace only (black), (*b*) none of the toxophorous group left.

Fig. 3. Diagram showing various forms of receptors or side-chains and various substances having the proper relation of outline to these receptors.

corresponding side-chains or receptors of the cell molecules. The side-chains of the food must have a specific relation to the side-chains of the cells, or as Emil Fischer has expressed it they must be adapted to one another as the key to the lock. The receptors, in addition to their normal function of assisting in the nutrition of the cells, possess fortuitously and incidentally the capacity of anchoring to themselves this or that toxin. One must here revert to Ehrlich's view (see p. 475) that the diphtheria toxin molecule consists of two groups of atoms (see Fig. 1), the toxophorous group (*t*), to which the poisonous properties of the toxin are due, and the haptophorous (ἅπτω = I seize) group (*h*), by means of which the connection between the cell or the antitoxin and the toxophorous molecule is established. It is by means of these haptophorous groups then that the toxin molecules become attached to the "side-chains" or receptors of the cells of the animal body (see Fig. 3). The haptophorous group of one toxin finds the suitable receptors in one kind of cell, the haptophorous group of another toxin in another kind of cell, and in this way we have an explanation of the specific action of toxins.

The first stage of toxin action is the anchoring of the toxin molecule by its haptophorous group on to the suitable receptor of a certain cell (see Fig. 4), and this likewise is the first stage in antitoxin production. The receptor while it is occupied in this manner cannot exercise its normal physiological function of conveying nutrition to the cell protoplasm. Thus there is created a defect in the cell-life which the cell endeavours to make good by a process of regeneration. Fresh side-chains are produced (see Fig. 5), and as these are occupied again and again by fresh injections of toxin the attempt at compensation gives rise to the production of an excess of side-chains in accordance with the principles enunciated by Weigert. The side-chains then being produced in excess of the requirements of the cell are thrown off into the blood stream where they constitute the antitoxin (see Fig. 6). In their free state in the blood stream they are still capable of union with the toxin, which is thus diverted from attacking the susceptible cells (see Fig. 7).

It follows from Ehrlich's view that the toxoid having the same combining power for antitoxin as the original toxin should also be capable of occupying the corresponding side-chains of the cells in the animal body, and should thus produce the necessary condition for the stimulation of the cell to the hyper-production of side-chains, that is, to antitoxin production. Ehrlich assumes throughout most of his work

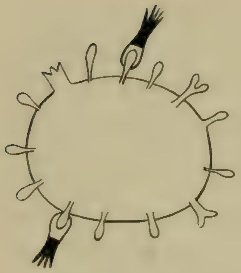


Fig. 4.

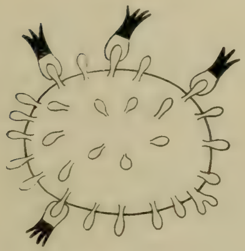


Fig. 5.

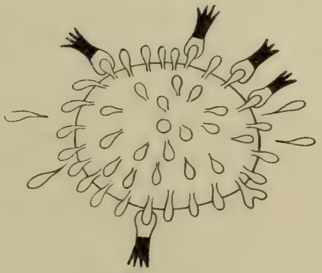


Fig. 6.

Fig. 4. First stage of antitoxin formation; toxin molecules anchored on to the suitable receptors.

Fig. 5. Second stage of antitoxin production. To compensate for the number of receptors thrown out of action owing to their being occupied by toxin molecules there occurs a production of side-chains in excess of the normal.

Fig. 6. Third stage. Hyper-compensation takes place so that a larger number of receptors is provided than is necessary for the cell life. These unnecessary side-chains are thrown off into the blood and constitute the antitoxin.

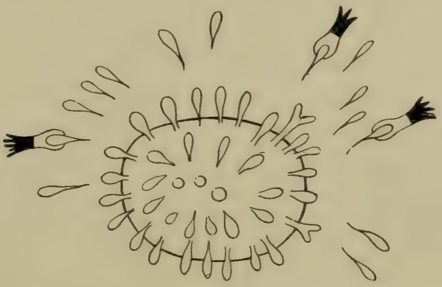


Fig. 7. Showing combination of free receptors, or antitoxin, with toxin molecules in the blood, and demonstrating the mechanism of cell protection by means of antitoxin.

that toxoids are capable of giving rise to the production of antitoxin.

It will be recalled that several of the methods suggested for the early stages of immunisation are based on the view that toxins modified by heating and chemical agents are still capable of stimulating the animal body to produce antitoxin. The question has been raised as to whether the mere occupation of the receptors is sufficient to stimulate the cell to antibody production. Von Dungern (1902) was the first to suggest that something of the nature of an irritative stimulus was necessary in addition to the occupation of the receptors. His view was largely based on the fact that during the ordinary nutritive processes nothing of the nature of an antibody or precipitin could be found in the blood. Ehrlich, Morgenroth (1902), and Wassermann (Brussels Congress, 1903) have, therefore, assumed that in addition to the occupation of the receptors there must be a stimulation or irritation of the cell, which is probably to be attributed in the case of toxic substances to traces of the toxophorous groups which have remained undestroyed. Bruck (1904) has further investigated this point in relation to tetanus toxin. He finds that tetanus toxoid, though it can combine with tetanus antitoxin, is not by itself capable of giving rise to the formation of antitoxin, but that if a mere trace of toxin is present antitoxin is produced. The mere occupation of the receptors by the haptophorous group is in this case not sufficient for antitoxin formation; the toxophorous group must be present to give the necessary stimulus to the cell.

The question as to whether diphtheria toxones can produce antibodies, and the nature of the latter (if any) is of great theoretical interest. The point was investigated by Madsen and Dreyer who found that toxones were capable of actively immunising the guinea-pig, rabbit, goat and horse, and that the serum in all cases, except in the rabbit, contained antitoxin. The toxones, therefore, have haptophorous groups which are closely allied to or identical with those of the toxins[1].

A remarkable confirmation of Ehrlich's theory was afforded by Wassermann (1898), who argued that, if Ehrlich's hypothesis was true, antitoxin in its pre-formed state ought to be capable of demonstration in the normal susceptible cells of certain organs, since certain toxins attack them in a specific manner. For example, we know that tetanus toxin attacks the cells of the nervous system, therefore in these cells antitoxin ought to be demonstrable. On applying the test of experiment to this view he was able to show that an emulsion of guinea-pigs' brain could fix the tetanus toxin and neutralise its action[2]. Roux (1898) argued that if the nervous system contained antitoxin toxin injected into it should be at once neutralised, and cause no toxic effects. He demonstrated, however, that the intracerebral injection of tetanus toxin causes the death of the animal, and that smaller quantities are necessary to produce a lethal effect by this method than by the ordinary methods of injection. He held that the result was not compatible with Ehrlich's theory. Ehrlich, however, has pointed out that Roux's results so far from being in opposition to his theory are confirmatory of it. The receptors, so long as they are attached to the cells, are the connecting links between the cells and the toxin, and are thus in a sense a danger to the cells, whereas, when they are thrown free into the blood stream, still possessing as they do the property of attracting the toxin molecules, they form a protection to the cells from which they divert the toxin. Weigert illustrates this relation by a reference to the action of a lightning conductor. The copper or iron rod if placed inside the building without a suitable earth connection would be an added danger, whereas, when insulated and separated from the building, it affords protection by conducting the electricity to earth.

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[1] Madsen's later view will be dealt with elsewhere.

[2] The interpretation of this experiment has led to much controversy, into the discussion of which it would be impossible to enter here.

CHAPTER XVII.

THE EFFECTS OF ANTITOXIN ON TOXIN.

Theories regarding the interaction between antitoxin and toxin. Further analysis of the method of action of the antitoxin on the toxin. Brief summary of Ehrlich's theory of the relations of antitoxin to toxin. The physico-chemical theory of Arrhenius and Madsen. Criticisms of the views of Arrhenius and Madsen. The adsorption views of Bordet. The behaviour of antitoxin in the animal body when used for the production of passive immunity. The disappearance of antitoxin from the blood. Absorption of antitoxin from the alimentary canal. The question of the production of anti-antitoxins. Inherited immunity. Transmission of inherited immunity. Antimicrobial diphtheria serum.

Theories regarding the Interaction between Toxin and Antitoxin.

Soon after the discovery of antitoxin the nature of the interaction between toxin and antitoxin became a subject of investigation and controversy.

Behring held that the antagonism was of a chemical nature, and that the antitoxin neutralised the toxin in the same way as an alkali neutralises an acid. On the other hand, Buchner maintained that the antitoxin did not act directly on the toxin but indirectly through the intervention of the cells. The controversy on the subject between Behring and Buchner, which took place in 1894, was soon followed by a series of investigations on the subject. The chief supporters of the chemical view were Behring and Ehrlich, the opposition in favour of the cellular and vital hypothesis was led by Buchner, Metchnikoff, Roux, and Calmette. The death-blow to the cellular hypothesis was given by Ehrlich, who by a beautiful experiment was able to demonstrate the neutralising action of antitoxin on toxin *in vitro* in the absence of all living cells.

Ricin, when brought into contact with a suspension of dead red blood corpuscles in a test tube, causes their agglutination; when, however, a suitable quantity of antitoxin is added, the agglutination no longer takes place. Ehrlich was further able to show that when antiricin was added to a solution of ricin, in sufficient quantity to inhibit its agglutinating power on red blood cells, its toxic action on animals was also abolished. He thus proved in this case that the neutralisation which could be demonstrated *in vitro* was valid *in corpore*.

Since Ehrlich's fundamental experiment a large number of similar test-tube experiments have become possible with the expansion of the knowledge of haemolytic and agglutinating agents of animal, vegetable and bacterial origin: we need only mention cobra venin (Stephens and Myers), abrin (Ehrlich), tetanolysin (Ehrlich and Madsen) as examples of a large group of similar substances which produce haemolysis or agglutination of red blood corpuscles.

The experiments carried out by Morgenroth on rennin, Dean and Achalme on trypsin, and Sachs on pepsin with their corresponding antibodies have satisfactorily demonstrated the neutralisation of toxin by antitoxin (ferment by anti-ferment) in the absence of all cellular elements.

In this connection also may be mentioned the precipitin experiments of Kraus, Tchistowitch and Bordet. These experiments afford conclusive proof that the action of the antitoxin on the toxin is direct and not through the intervention of the cells of the animal body.

Further analysis of the method of action of the antitoxin on the toxin.

Does the antitoxin combine with the toxin so as to convert it into an atoxic modification thus causing its destruction as toxin, or does it simply neutralise the toxin forming an atoxic combination by a process analogous to the neutralisation of an acid by a base with the resulting formation of a neutral salt? Calmette (1895) found that whereas cobra venin is not affected antivenin is destroyed by heating to 68° C. for 10 minutes. Mixtures in certain proportions of venin and antivenin produced no symptoms when injected into rabbits of a certain weight. Similar mixtures after standing 10 minutes, and thereafter being heated to 68° C. for 10 minutes before injection into rabbits of the same weight, rapidly caused their death.

Wassermann (1896) found that *pyocyaneus* toxin was not affected by temperatures which completely destroyed its antitoxin. By heating newly made neutral mixtures he was able to demonstrate that the toxin, uninfluenced by its association with the antitoxin, was still capable of killing animals. Both Calmette and Wassermann arguing from these experiments gave their support to the vital theory. Marenghi (1897) carried out analogous experiments with diphtheria toxin and antitoxin mixtures. In this case, however, it is the toxin which is destroyed by heating, the antitoxin remaining unaffected. It had been shown by Roux and Yersin that diphtheria toxin is destroyed at 60° C., whereas antitoxin is unaffected by temperatures up to 70° C. Mixtures of toxin and antitoxin, which were neutral for the guinea-pig, after being heated to temperatures sufficient to destroy the toxin, were demonstrated to contain free antitoxin since additions of fresh toxin were found to be neutralised.

Martin and Cherry (1898) pointed out that previous investigators had in their researches neglected the *time* factor, which may be a very important element in any possible chemical interaction between toxins and antitoxins. The experiments, for example, of Calmette and Wassermann can be repeated and their results confirmed, and yet by modifications of the factors, *time, temperature* and *active masses,* exactly opposite results may be arrived at, proving that their conclusions are unjustified. Martin and Cherry in their series of experiments on the diphtheria toxin and antitoxin combination employed the gelatin filter of Martin, which consists of a Pasteur-Chamberland filter impregnated with gelatin. Through such a filter a diphtheria toxin can be forced by a pressure of 50 atmospheres without undergoing any very marked loss in toxicity, whereas, as shown by Brodie, antitoxin owing to the fact that it possesses a larger molecule cannot pass through, but is held back along with the proteids. Neutral mixtures of diphtheria toxin and antitoxin, which had been allowed to remain in contact at 30° C. for two hours, were filtered through the gelatin filter. The filtrate was found to be innocuous to guinea-pigs even in quantities corresponding to 32 fatal doses. From this experiment the deduction is made that the smaller molecule of toxin must have entered into some sort of chemical relationship with the relatively large molecules of antitoxin, during their sojourn together, prior to filtration.

The value of the factors, time and influence of proportion of active masses, was shown by a series of experiments on the snake venin of *Hoplocephalus curtus,* and its antivenin. The mixtures of two, three and four lethal doses of venin with 1 c.c. of antivenin were allowed to stand at 20—23° C., for varying times, 2, 5, 10, 15, and 30 minutes, and then injected into rabbits. The results are shown in the following table:

Proportion of toxin to antitoxin per kilo. of body wt.		Control, venom only	Time allowed for interaction of toxin and antitoxin: temp. 20—23° C.					
Antitoxin	Toxin		2 mins.	5 mins.	10 mins.	15 mins.	30 mins.	Injected unheated ∞ mins.
1 c.c.	2 fatal doses	Died 15 hours	Lived (very ill) for 2 days	Lived (ill 1 day)	Lived (no symptoms)	Lived (no symptoms)	Lived (no symptoms)	Lived (no symptoms)
1 c.c.	3 fatal doses	Died 12 hours	Died 20 hours	Died 28 hours	Lived (ill 2 days)	Lived (ill 1 day)	Lived (no symptoms)	Lived (no symptoms)
1 c.c.	4 fatal doses	Died 9 hours	Died 13 hours	Died 15 hours	Died 23 hours	Lived (very ill 2 days)	Lived (no symptoms)	Lived (no symptoms)

In this connection Danysz' (1902) experiments on the action of gastric juice on neutral ricin-antiricin mixtures are of great interest. When ricin and antiricin are submitted to the action of gastric juice for 16 hours at 40° C., the antiricin is found to have been completely destroyed, whereas the ricin is only slightly attenuated. A mixture of ricin and antiricin is prepared so that it has no agglutinating action on the red blood corpuscles as tested *in vitro* and no toxic action as tested on guinea-pigs. When this neutral mixture has been digested in the manner indicated it is found that the proteolytic ferment, while destroying the antiricin, leaves the ricin but little influenced so that the mixture becomes agglutinating and toxic.

These experiments, while proving that something of the nature of a chemical union takes place between the toxin and antitoxin, clearly show that the antitoxin does not destroy the toxin, since it is possible to recover it unaltered from neutral mixtures.

While the majority of investigators now adhere to this view of a direct action of antitoxin on toxin, there is still great divergence of opinion as to the exact mechanism of the interaction, and after this preliminary statement the more important views will be dealt with under separate headings.

BRIEF SUMMARY OF EHRLICH'S THEORY OF THE RELATIONS OF TOXIN TO ANTITOXIN, AND ON THE MECHANISM OF THE PRODUCTION OF ANTITOXIN.

Ehrlich's theory, owing to the important influence it has had in the investigation of the whole subject of diphtheria immunity, has already been dealt with in various sections of this work. Notwithstanding that it entails a certain amount of repetition, it has, however, been considered desirable to give here a brief summary of the subject so that the argument may be presented as a whole.

Judging from the history of the ferments, whose close relationship to the toxins was early pointed out by Roux, we cannot hope for much help from the purely chemical side in elucidating the problems connected with toxins. At the same time the most intimate knowledge that we possess of the nature of toxins has been obtained by the use of physico-chemical methods. Ehrlich obtained many of the most important facts on which he bases his views of the constitution of diphtheria toxin by using the method of partial saturation of the toxin with the antitoxin. If a mixture of hydrochloric and acetic acids is gradually saturated with an alkali the bulk of this goes first to the acid of stronger avidity, the hydrochloric acid, and then afterwards to the weaker acetic acid. Ehrlich has found analogous facts when diphtheria toxin is partially saturated with antitoxin, and by graphic demonstration of the course of the neutralisation has shown that the constitution of diphtheria toxin is much more complex than was at first surmised, and that the toxin and its derivatives possess quite different affinities for the antitoxin. Similar results were obtained by a number of workers in regard to other toxins. Ehrlich was also influenced in the elaboration of his views by those of Emil Fischer on the atom groupings of ferments. Fischer found that only such substances as possess a group into which the ferment group fits as the key into the lock are acted on by a particular ferment, and that it is only through the relation of these two groups, the one belonging to the ferment, the other to the fermentable substance, that there is obtained the union of the two molecules which is essential for the chemical action.

Ehrlich, applying these views to the present question, holds that the specific relationship of toxin to antitoxin can be most simply expressed by the assumption of two groups which correspond in their configuration. He assumes that the toxin molecule contains two functionating groups: a haptophorous, and a toxophorous, group. In favour of the assumption of a toxophorous group was the wide experience which had been obtained of the relationship between chemical constitution and physiological action. Thus, it had been shown that certain physiological actions can only be ascribed to certain groups, for example, in a great number of hypnotics only the aethyl group, and in a great number of anaesthetics only the benzoic acid radicles are concerned. It was a natural inference, therefore, to assume in the case of the toxins a special group giving rise to the toxic condition, that is, a toxophorous group.

As a strictly chemical characterisation of the toxins is not possible the criteria of the toxin nature must be purely biological. Especially marked are the nature of the toxic action and the ability to produce specific antibodies in the animal organism. The toxins share this property with a number of other substances; some of them, like the ferments, more or less toxic, others, as the albuminous and cellular substances, quite harmless. The period of incubation following toxin administration cannot, in most cases, be curtailed beyond a certain time, however much the dose is increased. The latent period in most instances lasts from six to twenty-four hours but may extend, as in diphtheria, to 14—20 days. It is doubtful if latency is an essential characteristic of toxin action, since the closely allied venin, ichthyotoxin and haemotoxin can act without any incubation period. A much more definite characteristic is the capacity to furnish antitoxin, since this is not shared by substances possessing a well-defined chemical composition. Ehrlich failed to produce antisera for alkaloids and toxic glucosides, and also failed to confirm the view that tetanus antiserum has an antitoxic action against strychnia.

In his earlier work Ehrlich showed that such substances as the alkaloids and colouring materials are present in the tissues, not in the form of close chemical combinations, but only loosely anchored to the cells. Corresponding to the loose character of the combination is the short duration of the action of such bodies as antipyretics, compared to the long lasting injuries which the toxins produce, and which point to their having taken a firm hold on the protoplasm. Those substances which form a lasting combination with protoplasm and enter into its constitution have long been stated to be "capable of assimilation," and most of these bodies have been held to belong to the group of nutrient substances. Ehrlich holds that this capacity for assimilation must depend on a chemical combination. He assumes that for such union there must be present two atom groups which have an attraction for one another, and he, therefore, suggests that the protoplasm molecule must have side-chains or receptors which have a maximum chemical affinity for certain groups of the specific nutrient substances which are thus anchored to the cells. To make the essence of toxin action comprehensible is, as Ehrlich asserts, only a short step. It is enough to assume that the toxins which, in their origin and in their chemical properties, may be regarded as closely allied to the albumins, peptones, etc., also possess groups which correspond to those of the nutrient substances, and which have the capacity to combine with the receptors of certain cells. Such atom groups of the toxin molecule Ehrlich calls "haptophorous groups" because they produce the fixation on to the cells. This view has been strengthened by numerous observations which prove that quite a number of substances, which are not toxins but nutrient substances, produce in the animal organism genuine antibodies. Bordet, for instance, showed that milk, various forms of serum, etc., also produce antibodies, "precipitins," which precipitate the corresponding body in a specific manner. The chief evidence in favour of the two functions, the haptophorous and the toxophorous, is based largely on observations on hemitoxin, in which half of a toxin becomes converted into an atoxic substance or toxoid which has still the power of combining with the same amount of antitoxin. The easiest explanation of the phenomenon is that the toxoids possess haptophorous but not toxophorous groups.

With the help then of the haptophorous group the toxin molecule is anchored on to the cell. This, however, does not sufficiently explain the toxic action. The presence of the second already mentioned toxophorous group is necessary. It acts on the protoplasm by means of the intermediate action of the haptophorous group.

On such an hypothesis Morgenroth explains in the simplest manner the interesting observation, first made by Courmont, that frogs injected with tetanus toxin if kept in a cool place do not develop tetanus, but if placed in an incubator at 37° C., quickly become tetanic and die. That the toxin was already fixed to the cell was proved by injecting sufficient antitoxin to neutralise the toxin, and after sufficient time for union had elapsed placing the frogs in the incubator. In spite of the presence of the antitoxin they developed a fatal tetanus proving that the tetanus toxin had become fixed to the cells in sufficient quantity to cause a fatal tetanus.

Ehrlich also instances the fact, observed by Billinger and H. Meyer, that marmots and bats are not affected by tetanus toxin during their winter sleep, but succumb to its action on awaking. Both these interesting and anomalous observations may be explained on the hypothesis of two groups being present, the haptophorous groups being capable of becoming fixed on to the nerve cells at a lower temperature than is required for the action of the toxophorous groups.

The incubation period can be explained easily in this manner. The haptophorous group quickly combines with the cell, the toxophorous group comes more slowly into action. This is confirmed by the experiments of Dönitz and Heymans who showed that when tetanus or diphtheria toxin is injected into an animal it disappears very quickly from the blood, and must, therefore, be fixed to the tissues and organs. This disappearance from the blood occurs long before the onset of any signs of intoxication. That the toxin has become fixed on to the susceptible tissues is proved by the method of injecting antitoxins at various periods after the toxin, and ascertaining the interval of time after which the antitoxin is no longer capable of saving the life of the animal. The experiment was so arranged that when a certain quantity of serum was injected at the same time as the toxin its action was completely neutralised. After the lapse of six minutes the corresponding dose of serum was no longer capable of saving the animal's life, showing that some of the toxin had already become fixed on to the susceptible cells. Yet in such a case no symptoms would appear till 12 to 24 hours after the toxin injection. This is strong evidence in favour of the haptophorous group having become fixed on to the suitable side-chain long before the toxophorous group came into action. In the above experiment if multiples of the quantity of serum were employed the animal could still be saved, showing that the cells, to a certain extent, were capable of being freed from the toxin already anchored on to them[1].

The susceptibility of the organism to poisons depends, therefore, on the presence of suitable "receptors." Many forms of natural immunity and certain forms of acquired immunity may be ascribed to the fact that suitable receptors are absent or defective. That poisons such as diphtheria toxin only harm certain species of animals, such as mice, in very large doses, whereas others, such as guinea-pigs and rabbits, succumb to the minutest dose may be easily explained by assuming that the cells of the insusceptible animals do not possess the suitable receptors. If the receptors which have a particular affinity for a toxin are distributed widely through the tissues or organs of an animal's body, the dose of toxin necessary to kill such an animal will be greater than in the case of an animal in which the receptors are confined to one organ of great importance to life such as the brain.

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[1] H. Meyer's experiments showing that tetanus toxin is conveyed along the nerve trunks may require some modification of these views.

In the one case the toxin will have its activity diffused and will be diverted from important organs; in the other case, the toxic force will be concentrated on cells, an interference with whose action endangers the life of the animal. The large swellings in the connective tissue which diphtheria toxin, ricin, and abrin produce can only be explained by an anchoring of the toxin on to the subcutaneous connective tissue cells. In this connection it is interesting to note that the delicate connective tissue of the mouse is untouched by diphtheria toxin, whereas a hundredth or thousandth part of the dose produces in the guinea-pig an extensive necrosis of the skin. We may here recall Buchner's experiment wherein he observed that a mixture of tetanus toxin and antitoxin, which was harmless to the mouse, was still capable of causing tetanus in the guinea-pig. The probable explanation is that a certain amount of toxin remained unneutralised in the mixture, and Ehrlich suggests that in the body of the mouse there exist a number of receptors distributed through the body, whereas in the guinea-pig most of the receptors are confined to the nerve cells. This may also explain the fact that the guinea-pig is much more susceptible than the mouse. In this way it is easy to understand how the trace of unneutralised toxin, finding its way direct to the important nerve cells of the guinea-pig, manifests its toxic action.

Receptors in Production of Antitoxin.

If the receptors of a cell are occupied by toxin a defect in the cell life is produced and the affected side-chain can no longer exercise its normal function of taking up nutrient substances. According to Weigert's law this defect will not only be compensated but overcompensated. This over-compensation can, through continued injections of appropriate amounts, be carried so far that the receptors are produced in such abundance as to be no longer of value to the cells, and are thrown as useless ballast into the blood fluid. These thrown-off receptors, or side-chains, according to Ehrlich's theory, constitute the antitoxin, which when present in the blood stream can, by uniting with it, divert the toxin from the susceptible organs.

This theory explains the multiplicity of the antitoxins and their specific character on the basis of normal physiological processes. Corresponding to their origin the relation of the antitoxins to the toxins is of a purely chemical nature; by their union they form a new combination indifferent to the animal body as has been shown by Fraser, Ehrlich, Cherry and Martin, Behring and Knorr.

The Constitution of the Toxin.

Ehrlich became convinced that the toxins were more complex in their nature than had been generally assumed. When we consider what a large number of substances one cell can produce (the bark of the quinine plant alone produces about 20 different alkaloids, quinine, cinchona, cinchonidin, etc.), it seems improbable *à priori* that a bacterial cell should produce a single poison. We must in future reckon with the probability that the products of secretion of bacilli contain quite different forms of poison. Ehrlich, for example, showed that tetanus filtrates contain, in addition to the spasm producing poison, one which dissolves the red blood corpuscles. Through Ehrlich's, and later Madsen's, researches it was shown that tetano-spasmin and tetanolysin possess different haptophorous groups which fit different receptors. The two corresponding antibodies are produced together but in different proportions during immunisation.

A further question which arises is whether secretion products of bacteria which possess similar haptophorous groups are of identical nature. The investigation of diphtheria toxin convinced Ehrlich that this is not so, but that toxic varieties exist to which a common haptophorous group is suitable. Besides the typical toxin the diphtheria bacillus produces at the same time and commonly in like quantity a second product, "toxone," which is strongly differentiated from the chief poison by its action on the animal body. Through the researches of Madsen, Ehrlich, Woodhead and others we know that diphtheritic paralysis, the onset of which takes place long after the acute intoxication caused by the toxin has disappeared, is due to the action of the toxone. The toxone is also characterised by the fact that its local action is slighter than that of the toxin. It produces no necrosis but only slight transitory inflammation, which may be absent in the case of small doses.

It must be assumed that the toxone possesses the same haptophorous group as the toxin, since it is completely saturated by antitoxin. The relationship of the toxone-haptophorous group to the antitoxin is distinctly weaker than that of the toxin. If a mixture of both substances is partially saturated with antitoxin the toxins are first neutralised and later the toxones in a manner similar to the neutralisation of a mixture of hydrochloric acid and acetic acid by a base, in which case the hydrochloric acid is first saturated before any combination occurs with the acetic acid. The functional difference of the two bodies is probably due to the fact that the nature of their respective toxophorous groups is essentially different.

The toxones are not products derived from the toxins since in both experimental and clinical observations, where the living bacilli are concerned, toxone action is clearly to be seen, and, since we believe that the toxic products which are produced in the body are at once absorbed without having time to pass into modified forms, we must assume a primary production of the toxones, in favour of which assumption other purely clinical observations may be adduced.

Of greater importance are certain modifications of the toxin, namely toxoids. If the same amount of antitoxin, for example the "immunity unit," saturates in the case of one toxin a quantity which can kill 100 guinea-pigs, and in a second only the lethal dose for 15 guinea-pigs, it must be assumed that in the toxin broth other substances are present in addition to the toxin which have a specific affinity for the antitoxin. Prolonged examination of the loss of toxicity of a toxin gave accurate information in regard to the meaning of these substances. It was found that during the spontaneous weakening of a toxin the neutralisation constants L_0 and L_+ can remain completely unchanged, while the "absolute toxicity" goes down to a half or even lower. It follows from this that the toxophorous group only, and not the haptophorous group, has been damaged. We must, therefore, assume that the toxophorous complex is more easily affected by chemical or thermal injuries than the haptophorous, and that "toxoids," the substances which are non-toxic but are nevertheless capable of uniting with antitoxin, occur as the result of the changes produced by such influences.

It is probable that thermal and chemical agents, though capable of producing completely atoxic substances which retain the power of immunising animals, do not affect a substance of such complex structure as the toxophorous group uniformly, but may act in a graduated fashion leaving traces of toxic substances which may be demonstrated only on particular races of animals.

Toxoids and Toxones as Immunising Agents.

That toxoids can give rise to antitoxins is held by Ehrlich to be a strong support to his view that two groups, the haptophorous and the toxophorous, are present in the toxin. He holds, for example, that animals can be immunised against tetanus by the use of toxoids, and that the only easy explanation is the assumption that the haptophorous group has remained intact and capable of occupying the side-chains of the antitoxin-producing cells, which are thus stimulated to elaborate and throw off fresh side-chains, in other words to produce antitoxin.

THE PHYSICO-CHEMICAL THEORY OF ARRHENIUS AND MADSEN[1].

Arrhenius and Madsen have recently brought forward another theory of the constitution of toxin and of the nature of the combination which takes place between toxin and antitoxin. They hold that the assumption of such a complex structure for diphtheria toxin as postulated by Ehrlich is unnecessary. They base their views on a number of observations obtained by using physico-chemical methods of investigation: in reality, by an extension of the methods of investigation which were first introduced into the subject with such conspicuous ability by Ehrlich.

Ehrlich, as we have seen, came to the conclusion that the union between toxin and antitoxin was similar in nature to the union between a strong acid and a strong base. Starting his argument from that point he arrived at the conclusion that diphtheria toxin was built up of a number of substances of greater or less toxicity and having a greater or less affinity for antitoxin. Arrhenius and Madsen hold in opposition to this view that the union between toxin and antitoxin resembles more the union between a weak base and a weak acid. The following is a very brief outline of their argument.

When increasing quantities of a strong acid are added to an aqueous solution of a base the basic quality of the solution gradually diminishes, and finally disappears when the equivalent quantity has been added. Thus if one equivalent of acid be added to four equivalents of base three equivalents of base remain free; if three equivalents of acid be added to four equivalents of base one equivalent of base remains unneutralised. In other words, the amount of base neutralised is proportional to the amount of acid added and this relation can be plotted graphically as the straight line *AN* in Fig. 8 where the ordinates represent the base and the abscissae the acid equivalents.

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[1] I am much indebted to Mr J. A. Craw for revising the proofs of the sections on the Physico-chemical and Adsorption theories.

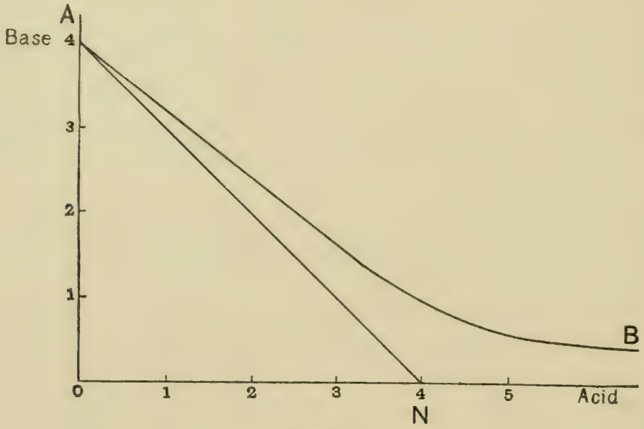

Fig. 8.

This state of affairs strictly holds only for strong acids and strong bases; in the case of weaker acids and weaker bases there is a tendency to the formation of a curve near the neutralisation point *N* where the neutralisation line *AN* passes on to the axis of the abscissae. In reality, even in the case of strong acids and strong bases a slight bending occurs at *N*, but is so slight that it cannot be detected by ordinary chemical means. There is only a quantitative difference between strong acids and strong bases on the one hand and weak bases and weak acids on the other hand.

If the base and the acid are both very weak the bend near *N* becomes very distinct, *e.g.*, the neutralisation of ammonia by boric acid may be represented by the curve *AB*.

As will be seen from the curve (Fig. 9), after the addition of the first equivalent of the boric acid 50 % of the ammonia is neutralised; after the addition of the second equivalent 66·7 %; after the addition of the third equivalent 75 %; and after the fourth equivalent 80 %. Therefore, whereas the first equivalent added neutralised 50 %, the second neutralises only 16·7 %, the third 8·3 %, and the fourth 5 %.

Arrhenius and Madsen carried out a series of haemolytic experiments using ammonia in place of a toxin, such as tetanolysin, and boric acid in the place of the antitoxin, and they found that the results were in every way comparable. By the method of partial saturation of the ammonia with boric acid they found that the first additions of the acid produced a much greater relative diminution in the haemolytic action than did the later additions, just as occurs in the case of tetanus toxin and antitoxin. The curve obtained by plotting out the quantities of uncombined ammonia as tested by their haemolytic action is seen in Fig. 10, and gives an almost exact graphic representation of what occurs in the case of the neutralisation of tetanolysin by anti-tetanolysin. The curve is of the same nature as that representing the equilibrium between a body in partial dissociation and the products of dissociation, in which case the condition of equilibrium has been found to be capable of expression by the equation:

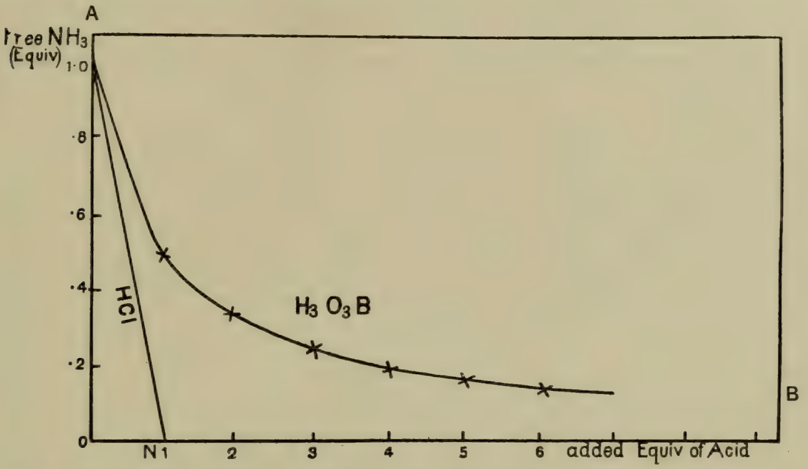

Fig. 9. Curve of Neutralisation of Ammonia by Acids (after Arrhenius and Madsen).

$$\text{(concentration of free acid) (concentration of free base)} \\ = K\,\text{(concentration of combined base)}^2,$$

where K is the constant of dissociation. In the cases of strong acids and strong bases $K=0$ (or more correctly is of exceedingly small magnitude).

Arrhenius and Madsen say that if Ehrlich's reasoning were applied to this experiment the first amount of ammonia neutralised would be regarded as three times as toxic as the second, the second twice as toxic as the third, and so on. Therefore ammonia is not a simple body but a complex one consisting of several components of different toxicity and different chemical affinities, a *reductio ad absurdum.* The toxin and the ammonia must be equally regarded as simple chemical substances, and the union between toxin and antitoxin, just as that between ammonia and boric acid, must conform to the law of chemical mass-action of Guldberg and Waage, viz., that the amount of the action is proportional to the active mass of each of the reacting substances.

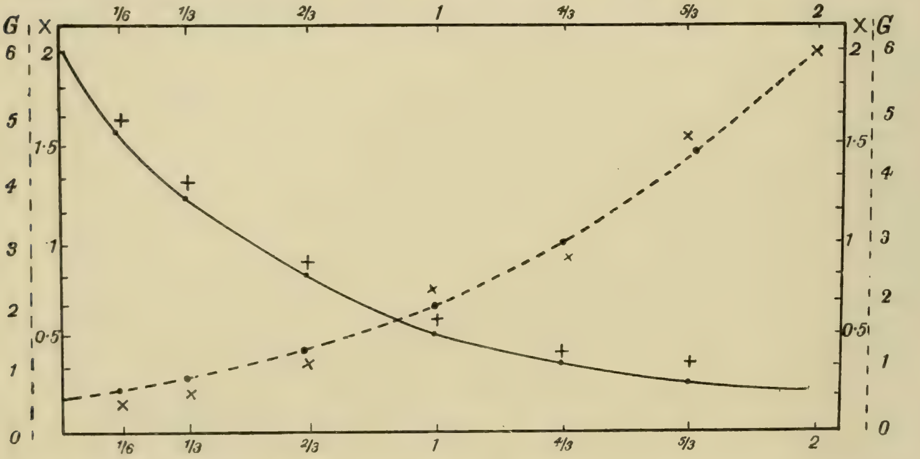

Fig. 10. Curves showing the Neutralisation of the Haemolytic Action of Ammonia by Boric Acid (after Arrhenius and Madsen).

Calculated values plotted as curves :—

+ observed values.

× ,, ,,

—— toxicity of mixture due to unneutralised ammonia (Haemolysin, *G*).

- - - - quantity of mixture required to produce a certain arbitrary haemolytic effect (X).

A series of mixtures are prepared containing an arbitrary quantity of ammonia (toxin) and increasing quantities of boric acid (antitoxin).

The toxicity of the mixture (*G*) is thus gradually diminished. *X* is the quantity which it is necessary to remove from such a mixture to produce, when introduced into a fixed volume of standard suspension of red blood corpuscles, a certain arbitrary haemolytic effect. From this it is clear that *X* will be inversely proportional to *G*, as is indicated by the two curves.

The view that toxins are of complex character resulted from the difficulty that attached to the investigation of the first toxin studied, namely, diphtheria toxin. A specially misleading feature in this case was the fact that apparently two forms of intoxication occurred, an acute form resulting in rapid death, and a chronic form resulting in late paralysis. A perfectly natural deduction was that there were present in the diphtheria toxin two separate substances. The second form of intoxication resulted especially when toxin together with a comparatively large amount of antitoxin was present and when such a mixture, even in large doses, failed to produce the acute toxic action.

Diphtheria toxin examined in this new light gives results which are quite in accordance with those obtained in the cases of ammonia and boric acid and tetanolysin and anti-tetanolysin. We can examine a toxin by adding varying quantities of antitoxin to an arbitrary quantity of the toxin and ascertain the toxicities of the resulting mixtures. In such experiments volume may be neglected as the volume of the body of the guinea-pig is practically constant. Just as in the case of ammonia and boric acid, the results may be represented by a curve, which has a close resemblance to the analogous curve of tetanolysin and its antitoxin, in which the ordinates indicate the toxicity of the resulting mixture, or the number of lethal doses in each, and the abscissae the amounts of antitoxin added to the constant quantity of toxin. By proceeding in this way Arrhenius and Madsen found that their results could be expressed by the formula

$$\frac{\text{toxin free}}{\text{vol.}} \times \frac{\text{antitoxin free}}{\text{vol.}} = K\left(\frac{\text{toxin-antitoxin}}{\text{vol.}}\right)^2$$

which had been found to apply to the case of equilibrium between a body in partial dissociation and its products of dissociation.

The equation for this case takes the form

$$\frac{1}{T_0}\left\{np\frac{1}{T}-\left(\frac{1}{T}-\frac{1}{T_0}\right)\right\}=K\left(\frac{1}{T}-\frac{1}{T_0}\right)^2,$$

where K is the dissociation constant which is different for each toxin; n is the quantity of antitoxin added in fractions of an antitoxic unit; T_0 is the number of lethal doses in the quantity of toxin employed where no antitoxin has been added, therefore $\frac{1}{T_0}$ is the minimal lethal dose of that toxin; T is the number of minimal lethal doses in a mixture after the addition of antitoxin, therefore $\frac{1}{T}$ is the minimal lethal dose of that mixture; p indicates the number of toxin equivalents in an antitoxic unit if we take the quantity of toxin employed as an arbitrary unit. K and p are determined from two or more equations of the above form where they are regarded as the two unknowns, the other values in the equations having been ascertained by experiment.

The manner of arriving at the above equation is as follows :

If we inject, for example, a quantity of the mixture sufficient to kill a guinea-pig on the fourth day, in which case the free toxin is equal to 1 M.L.D. or $\frac{1}{T_0}$, then $np\frac{1}{T}$ is the total quantity of antitoxin in the mixture, for $\frac{1}{T}$ is the quantity which contains 1 M.L.D. of the toxic mixture ; n of antitoxin was added, therefore $\frac{n}{T}$ is present before combination. Assume that one antitoxic unit is equivalent to p toxic units expressed in M.L.D., then the total amount of antitoxin, expressed as equivalent units, in the lethal mixture is $\frac{np}{T}$. The difference between the quantity added $\frac{1}{T}$, and the quantity free $\frac{1}{T_0}$, that is $\frac{1}{T}-\frac{1}{T_0}$, expresses the quantity of toxin fixed in combination. The basis of the whole theory is that toxin and antitoxin combine in equivalent proportions, therefore the combined antitoxin can also be expressed by $\frac{1}{T}-\frac{1}{T_0}$. The total antitoxin minus the fixed antitoxin gives the free antitoxin, therefore from the above determinations $np\frac{1}{T}-\left(\frac{1}{T}-\frac{1}{T_0}\right)$ expresses the free antitoxin.

Then the equation

$$\left(\frac{\text{toxin free}}{\text{vol.}}\right)\times\left(\frac{\text{antitoxin free}}{\text{vol.}}\right)=K\left(\frac{\text{toxin-antitoxin}}{\text{vol.}}\right)^2$$

can be expressed thus

$$\frac{1}{T_0}\times\left\{np\frac{1}{T}-\left(\frac{1}{T}-\frac{1}{T_0}\right)\right\}=K\left(\frac{1}{T}-\frac{1}{T_0}\right)^2.$$

A few examples of the experiments required to arrive at the necessary data may help to elucidate the matter.

A toxin has 420 M.L.D. in the quantity of toxin employed expressed as unity.

In this case no fraction of the antitoxic unit has been added, *i.e.* $n=0$ and $T_0=420$, so that $\frac{1}{T_0}=\frac{1}{420}=\cdot0023$. We may tabulate the result thus :

n	T_0	$\frac{1}{T_0}$
0	420	·0023

Now if ·1 c.c. of an antitoxic unit is added to the toxin and the toxicity of the mixture tested on guinea-pigs and it is found that there are only 306 M.L.D. in the mixture and 1 M.L.D. is $\frac{1}{306}$ or ·0032 the result may be tabulated thus :

n	T	$\frac{1}{T}$
0·1	306	·0032

In another experiment where ·2 c.c. of the antitoxic unit is added only 209 M.L.D. are present in the mixture, therefore

$$T=209 \text{ and } \frac{1}{T}=\frac{1}{209}=\cdot0047,$$

which may be tabulated thus:

n	T	$\frac{1}{T}$
0·2	209	·0047

By substituting these values which have been ascertained experimentally we have the data for two equations

$$(1)\quad \cdot 0023\,\{\cdot 1 \times p \times \cdot 0032 - (\cdot 0032 - \cdot 0023)\} = K\,(\cdot 0032 - \cdot 0023)^2,$$

$$(2)\quad \cdot 0023\,\{\cdot 2 \times p \times \cdot 0047 - (\cdot 0047 - \cdot 0023)\} = K\,(\cdot 0047 - \cdot 0023)^2,$$

from which the values of p and K can be determined mathematically.

Where only two such equations are employed to ascertain the values of p and K these will be only approximate, but the more equations, based on experimental data, employed the more accurate will be the values. p and K are therefore determined by Arrhenius and Madsen from as many data as possible and at the same time the corrections of the M.L.D. as ascertained from loss of weight and lethal period are employed (see p. 470).

The most accurate values possible for p and K having been determined the equations are then employed to arrive at the theoretical values of T.

A complete series of experiments by Madsen of the nature indicated are shown in the Table.

Neutralisation of Toxin No. 471 (*Arrhenius and Madsen*). *Ehrlich's Standard Test Serum was employed.*

$p = 2{\cdot}5$ $\qquad K = 0{\cdot}0122$

n	$n \times p$	T	Observed	Calculated
0	0	420	100	100
0·05	0·125	313	74·4	87·5
0·1	0·25	306	72·8	75·1
0·15	0·375	242	57·6	62·7
0·2	0·5	209	49·8	50·6
0·25	0·625	135	32·2	38·6
0·3	0·75	118	28·0	27·3
0·35	0·875	72·5	17·2	17·5
0·4	1·00	46·5	11·1	9·9
0·45	1·125	23·5	5·6	6·0
0·5	1·25	5	1·2	4·1

Note. K is the dissociation constant. T = the toxic units or minimal lethal doses in the mixtures. n is the fraction of an antitoxic unit which is added to the toxin. The constant quantity of toxin employed in these experiments being taken as unity, the value "p" indicates the number of equivalents of antitoxin contained in one antitoxic unit—

$$\frac{p}{1} = \frac{\text{immunising unit}}{\text{toxic unit}}.$$

If n parts of the antitoxic unit be added to this unit of toxin then the product "np" indicates the ratio between the equivalents of antitoxin and toxin in the individual cases examined

$$\frac{np}{1} = \frac{\text{equivalents of antitoxin}}{\text{toxin unit}}.$$

In the 4th and 5th columns under the bracket, the calculated and observed values of T are reduced to percentages.

The numbers obtained by experiment and calculation were found to be in agreement within the experimental error.

In the accompanying chart the observed and calculated values reduced to percentages from the above table are plotted. The ordinates show the number of lethal doses, the abscissae the amounts of antitoxin added to the constant quantity of toxin employed.

Chart showing the percentage values of T, observed values indicated thus ⊙, calculated values thus ● on the curve.

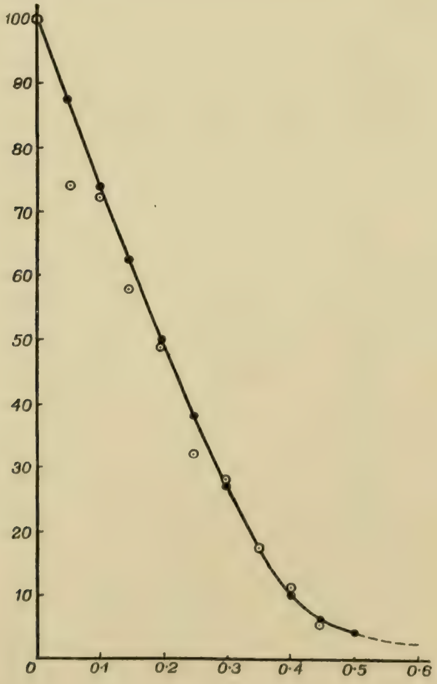

Fig. 11. The ordinates indicate the number of lethal doses in the mixtures (T), the abscissae the fractions of a c.c. of antitoxin added.

Toxicity of mixtures of Diphtheria Antitoxin and Diphtheria Toxin at Different Dates.

$p=2{\cdot}5$ $K=0{\cdot}0122$.

		T. observed (Feb. 1902)		T. observed (Sept. 1903)		T. calculated
n	$n \times p$	100	T.	100	T.	100
0·10	0·25	72·8	306	75·1	161	75·1
0·15	0·375	57·6	242	62·6	134	62·7
0·20	0·5	49·8	209	47·6	102	50·6
0·25	0·625	32·2	135	45·8	98	38·6
0·30	0·75	28·0	118	25·9	55·5	27·3
0·35	0·875	17·2	72·5	17·3	37	17·5
0·40	1·00	11·1	46·5	9·6	20·6	9·9
0·45	1·125	5·6	23·5	5·3	11·4	6·0
0·50	1·25	1·2	5·0	3·1	6·5	4·1
0·60	—	—	—	1·6	3·5	2·6

The toxin employed lost half its strength in 17 months though it maintained its power of fixing antitoxin, and the value of K remained quasi-constant. To explain this phase Madsen and Arrhenius have admitted, following Ehrlich, that the poison has been transformed into an inoffensive modification, "syntoxoid," possessing the same affinity for antitoxin, and the constant of equilibrium of the initial poison. If we take this view of the matter we must admit that the two molecules which result from the reactions of the toxin and of the toxoid with the antitoxin are different.

The reaction recalls that of etherification:

$$\text{acid} + \text{ethylic alcohol} \leftrightarrows \text{water} + \text{ether.}$$

If this is the case the reaction resembles an etherification in which two acids having the same affinity for an alcohol react on it, and give rise to two different ethers and water. The analogue of the water, the common substance resulting from the reaction, they call titoxin; of the analogues of the ethers, the one resulting from toxoid they call toxoidane, the other resulting from toxin they call toxinane.

$$\text{Toxin and antitoxin} = \text{toxinane} + \text{titoxin.}$$
$$\text{Toxoid and antitoxin} = \text{toxoidane} + \text{titoxin.}$$

From the formula it appears that one molecule of toxin combines with one molecule of antitoxin to form two molecules of the new combination toxin-antitoxin. If to a given quantity of toxin there is added a relatively small amount of antitoxin this will be almost entirely fixed and the surplus of the toxin will remain free. There will be almost no antitoxin free because in these proportions the combination toxin-antitoxin is only very feebly dissociated. As we increase the amount of antitoxin a greater amount of toxin will be fixed, but at the same time the toxin-antitoxin will be dissociated more and more, so that there will always be present toxin and antitoxin side by side. If incomplete association of the combination toxin-antitoxin did not take place then the union between toxin and antitoxin would be in the form of a straight line as in the case of the strong acid and the strong base.

As we add more and more antitoxin the quantity of the free toxin diminishes but the decrease occurs progressively more slowly, and theoretically however much antitoxin may be added there remains always some toxin free. This phenomenon is indicated by the form of the curve. The difficulty of determining the L_0 and the point of complete neutralisation can be explained from this point of view since such a neutral point does not exist. In this way also the toxone-zone of the spectrum may be explained. The slight oedema and late paralysis, according to Arrhenius and Madsen, are due not to a separate body toxone but to toxin either unneutralised or dissociated.

The dissociation of the toxin-antitoxin compound gives a comparatively easy explanation of the results obtained by Dreyer and Madsen in the rabbit and guinea-pig. A mixture of toxin and antitoxin, which had no effect on the guinea-pig, when injected intravenously into the rabbit produced paralysis; and a mixture, which in the guinea-pig gave rise to paralysis only, was rapidly fatal to the rabbit. It is difficult to understand this result if we regard the paralysing substance, the toxone, as a separate body, whereas it is easy to understand and interpret if we attribute both acute death and paralysis to the toxin and regard the difference as only a quantitative one.

Madsen and Walbum (1904) have obtained with neutral mixtures of diphtheria toxin and antitoxin results which they regard as an absolute proof of the occurrence of dissociation. They pour on to the surface of a column of gelatin a mixture of diphtheria toxin and antitoxin, which is absolutely harmless to a guinea-pig. After remaining for 66 days at a temperature of 2° to 3° C., half a c.cm. of the column of gelatin is cut off, melted and mixed with a lethal dose of toxin. This mixture injected into a guinea-pig does not kill the animal. Some antitoxin, therefore, has diffused into the upper layers of the gelatin. The 10 c.cm. below this upper layer is melted and one half of it when injected into a guinea-pig kills the animal; the

Chart showing the theoretical curve of neutralisation of diphtheria toxin by increasing quantities of antitoxin, contrasted with certain experimental data (after Madsen, 1903, *p.* 640).

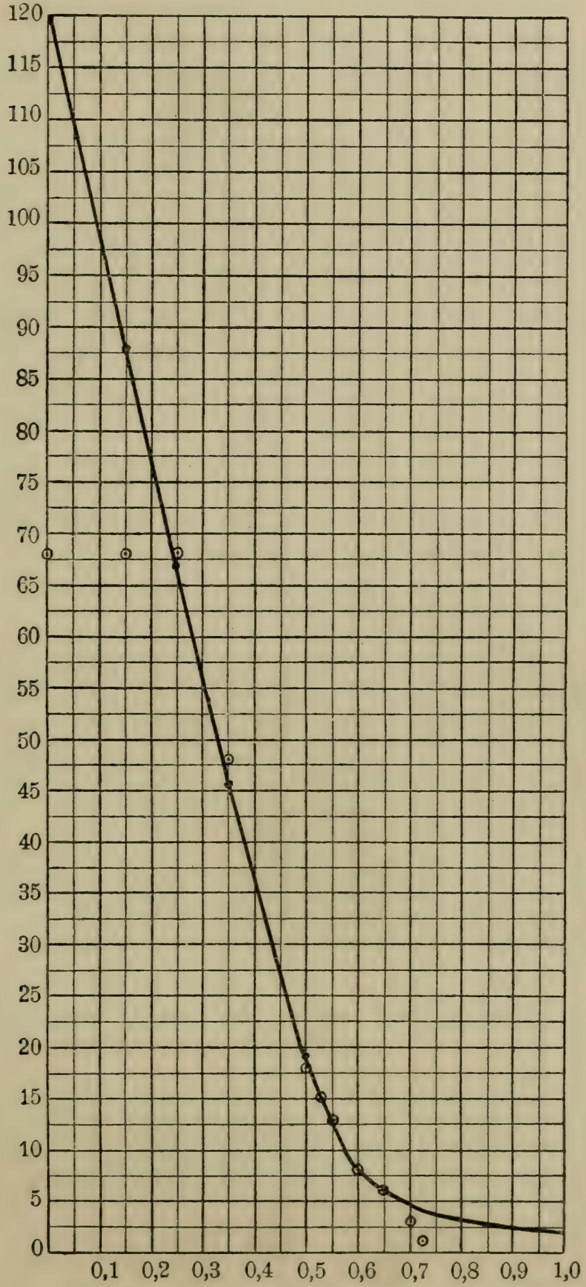

Fig. 12. The ordinates indicate toxicity or number of lethal doses, the abscissae the amounts of antitoxin added to the constant quantity of toxin.

● = calculated values. ⊙ = observed values.

other half, mixed with antitoxin, produces in another guinea-pig only a slight transient oedema, probably due to the gelatin. The interpretation of this experiment appears to be that a neutral mixture of diphtheria toxin and antitoxin has become dissociated, part of the dissociated antitoxin has diffused into the upper layer of gelatin, and the dissociated toxin being more diffusible has extended deeper into the gelatin.

By a similar method the constants of diffusion for diphtheria toxin and antitoxin, and for tetanolysin and anti-tetanolysin, were estimated with the result that the constants for the antitoxin were found to be much smaller than those for the toxins, indicating that the molecular weights of the antitoxins are much greater than those of the toxins.

Certain of the diphtheria curves worked out by Madsen do not coincide throughout their whole length with the theoretical curves. The upper part of the curve is quite flat and runs parallel to the axis of the abscissae (see Figs. 12 and 13).

Chart showing the toxicities of the mixtures obtained by gradually adding increasing quantities of antitoxin to a fixed quantity of diphtheria toxin (*after Madsen*, 1903, *p.* 639).

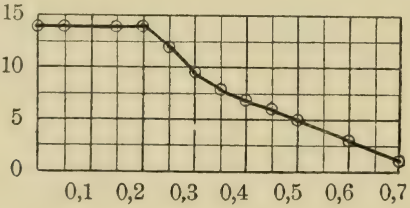

Fig. 13.

This indicates that during the process of partial saturation the first additions of antitoxin to a fixed quantity of toxin have no effect in diminishing the toxicity. Such a result could be explained by retaining Ehrlich's "Protoxoids," but in their more recent communications Arrhenius and Madsen state that in the case of certain toxins this appearance of the curve is absent and that it was probably due to an experimental error.

Arrhenius and Madsen have summed up their results and views in regard to diphtheria toxin and antitoxin as follows:

1. The neutralisation of diphtheria toxin by antitoxin follows the law of Guldberg and Waage.

2. Its constant of dissociation is comparatively insignificant, it varies in the observed cases between 0·004 and 0·03.

3. A toxin in losing its toxicity by age probably follows the law of the rate of monomolecular reactions.

4. That rate of reaction can be very different in different cases so that the stability of one toxin can be double that of another.

5. By its loss of toxicity the toxin does not at first lose to an appreciable degree its power of fixing antitoxin nor does it change its constant of dissociation.

6. No indications of the presence of protoxoids have been found although they have been purposely looked for.

7. It is no longer necessary to believe in the presence of toxones; nevertheless, concentrated solutions of toxin show perturbations due probably to a marked fall in the rapidity of reaction in the presence of high concentrations of antitoxin.

8. All these characteristics of diphtheria toxin can be explained on the following hypotheses:

A. Diphtheria toxin is a homogeneous toxin.

B. Diphtheria toxin changes slowly after the laws of monomolecular reactions into the atoxic body, toxoid.

C. The quantity of toxoid formed by a given quantity of toxin is here considered as equivalent to the toxin. Toxins and toxoids react in equivalent quantities with equal quantities of antitoxin, so that from one molecule of toxin or of toxoid and one molecule of antitoxin there are formed two molecules, the one of a body which is alike in the two cases (called "titoxine"), the other of a body which is not identical in the two cases, called "toxinane" when originating from toxin, and "toxoidane" when from toxoid. Like antitoxin these products are atoxic.

D. The constant of equilibrium is equal for the two reactions, namely, those of the toxin and of the toxoid with the antitoxin.

CRITICISMS OF THE VIEWS OF ARRHENIUS AND MADSEN.

In replying to these views Ehrlich (1903, 1904) adheres to his previous position. He reviews a number of the facts and analyses many of the data of which support his views of the complex composition of diphtheria toxin, and of the nature of the union which takes place between toxin and antitoxin.

In regard to the complexity of diphtheria toxin he says that this falls into line with a common biological experience, that organisms never produce a single characteristic substance but tend rather to give rise to a series of bodies which resemble one another. He instances opium which contains nearly 20 alkaloids, morphine, kodeine, etc., which have a certain similarity of action, but which are sharply differentiated from one another. Cinchona bark furnishes another good example.

He reviews his grounds for considering toxone a separate poison, secreted as such by the bacillus and not derived secondarily from the toxin. The striking difference in the time at which the action occurs and the widely different effects produced on the animal body are strongly in favour of there being two distinct substances at work. There is first the toxin effect which produces death within a few days or in sub-lethal doses gives rise to rapid local symptoms of inflammation in the subcutaneous tissue, oedema, alopecia, accompanied by loss of weight, etc. If on the other hand a man or animal has survived an attack of diphtheria, even if the local effect has been of the slightest, there may supervene after an interval of weeks characteristic paralyses. These late effects are of such a different character that Ehrlich holds they strongly support the view that they are produced by another poison, namely, the toxone.

Further, he refers to his observation that old diphtheria toxin, although its toxicity may have greatly diminished, still requires as much antitoxin as formerly to neutralise it. This can only be explained by the conversion of a part of the toxin into a less toxic substance, "toxoid," which retains its original avidity for antitoxin. The validity of this last argument has, indeed, been admitted by Arrhenius and Madsen. Ehrlich's analyses of the results obtained from the examination of the same toxins at different periods, and of toxins of widely different composition, are too elaborate for reproduction here and we must refer the reader to his original papers and those of Morgenroth (1904).

In addition to the biological arguments against the view that toxins are simple bodies, certain criticisms have been raised touching the physico-chemical views advanced by Arrhenius and Madsen.

If Arrhenius and Madsen's view, that the union between toxin and antitoxin resembles an etherification, is correct, then the toxin-antitoxin reaction must be reversible, for this is the essential feature of such a reaction; the acid acts on the alcohol and gives rise to ether and water; the water again reacts on the ether, and alcohol and acid result.

In such reversible reactions when the same quantities of the reacting substances are employed, no matter how they are brought together, the same equilibrium is established, that is to say, at the end of the reaction the same amounts of the free substances and of the products of reaction are always present in the mixture. Is the equilibrium of such a character in the toxin-antitoxin reaction as to indicate that the reaction is reversible?

Von Dungern (1904), reverting to an observation made by Danysz (1902), investigated this point. Danysz had found that it was not a matter of indifference whether the toxin was added altogether or in fractions to the antitoxic unit. If the L_0 dose of toxin is added to the unit and left in contact for six hours and then more toxin is added it requires a smaller addition of toxin to reach the L_+ dose than if all the toxin were added at once to the antitoxic unit. The difference between the L_+ and the L_0 is smaller, therefore, in the case of fractional addition. Von Dungern confirmed this fact for diphtheria toxin, and indicated its importance as an argument against the reaction being a reversible one and in favour of Ehrlich's assumption of the existence in diphtheria toxin of groups, having different avidities for the antitoxin.

The results obtained by this line of experiment also indicated that a closer binding of the combination occurred after union had taken place. The experiments of Danysz and von Dungern were repeated and confirmed by Sachs in the case of tetanus toxin.

Nernst (1904) has submitted some of the calculations of Arrhenius and Madsen to a searching examination. He finds that the selection of the figures in estimating the end points of reactions has been altogether arbitrary. The equilibria which they assume to exist in the case of toxin-antitoxin are not true equilibria but belong to the class of "false" equilibria, well known to the physical chemist. The variation of the dissociation constant from one diphtheria toxin to another seems to him to demonstrate the complexity of the toxic fluid. He concludes that the law of mass action (Guldberg and Waage) has been improperly applied. This law has hitherto been limited to gases and to true solutions, and this attempt by Arrhenius and Madsen to apply it to substances which are of the nature of colloidal solutions is not justified. In the case of toxins and antitoxins we are dealing not with true solutions, but with a form of suspension, the physico-chemical knowledge of which is only in its infancy. The physical chemist must have a more accurate knowledge of the laws governing the colloidal state before he can hope to deal successfully with biological problems such as the nature and relation of toxin and antitoxin.

Sachs (1904) discusses the question of reversibility. He refers to Martin and Cherry's work in which it was shown that although toxin alone passes through a gelatin filter no toxin was found to pass from a toxin-antitoxin mixture, showing that no free dissociated toxin was present in the mixture. He holds that Madsen and Walbum's experiment of the diffusion of toxin into gelatin is completely upset by Morgenroth's (1904) observation that 24 hours are required for complete union of toxin with antitoxin. Madsen and Walbum say in regard to their experiment that it is a necessary condition for dissociation that the mixture of toxin and antitoxin should be fresh and should not have stood long. Madsen and Walbum's experiment is only an additional proof, therefore, of the irreversible nature of the combination.

THE ADSORPTION VIEWS OF THE TOXIN-ANTITOXIN REACTION.

Bordet (1901—3) thinks that the fixation of toxin by antitoxin is a process analogous to the fixation of a dye on a tissue. He states that his view has been misunderstood, and that by such fixation he does not mean mere mechanical action to the exclusion from the reaction of all chemical characteristics. He holds it would be hazardous to assume, as some of his critics do, that the phenomena of coloration never come within the range of chemistry, but are purely mechanical depending on physical properties, such as the texture and porosity of the stained material. He instances the absorption of iodine from a watery solution by starch.

He holds that the reaction differs from ordinary chemical reactions in that the toxin and antitoxin by their union do not always form one and the same substance which invariably possesses the same characters. The toxin and antitoxin unite in variable proportions, and the molecules of antitoxin distribute themselves on all the toxic molecules present. A complex is thus formed which is less toxic. The phenomena of intoxication in the case of such toxins modified by antitoxin will not be the same as those resulting from the action of intact toxins.

Between the extremes of pure toxin and toxin entirely saturated all the transition stages of progressive attenuation can be conceived.

Every time the same proportions are used the same degree of toxicity of the compound will be attained.

Bordet thinks that a number of anomalous phenomena can thus be explained. It is easy to understand from this view how a mixture neutral for one species of animal may be toxic for another. The toxone phenomenon can also be explained on this hypothesis of compounds having different toxic actions.

Danysz (1902) holds a view identical with Bordet's, and in support of it has added interesting experimental evidence, to which reference has already been made.

Nernst (1904) pointed out that, since the substances involved in the reaction are both colloidal in character, the union is probably of the nature of an adsorption of the smaller toxin molecules by the larger antitoxin molecules.

Craw, J. A. (1905), in a series of experiments on megatheriolysin and antilysin, came to the conclusion that the removal of lysin from a solution by antilysin is not capable of interpretation as a purely chemical change, and that the law of chemical mass action does not apply when the lysin is present in excess. Lysin and antilysin in the free state are present both in sub-neutralised and over-neutralised mixtures. He holds that the phenomena are more analogous to certain adsorption phenomena.

THE BEHAVIOUR OF ANTITOXIN IN THE ANIMAL BODY WHEN USED FOR THE PRODUCTION OF PASSIVE IMMUNITY.

When diphtheria antitoxin is injected subcutaneously with the view of rendering an animal immune, a certain time must elapse before the maximum effect is attained. Owing to the large size of its constituent particles the antitoxin diffuses somewhat slowly, first into the lymphatics and thence into the blood stream. So slow is the passage from the site of inoculation into the general circulation that, according to Behring (1901), eight hours can be saved by injecting the antitoxin directly into the blood stream. Henderson Smith (1907) found that after a subcutaneous injection of diphtheria antitoxin the maximum antitoxic value of the blood serum was not reached till about the third day. Apart from its specific action, by means of which the recipient of the serum is rendered immune to the disease, certain secondary collateral effects are sometimes produced which will be dealt with in Chapter XIX.

When the antitoxin is injected into persons already affected with diphtheria it combines with any toxin which may be present in the blood and prevents the poison from attacking the susceptible cells. So long as there is free antitoxin in the blood the toxin elaborated at the site of infection and absorbed into the blood does not reach the cells for which it has a specific affinity, but is arrested and neutralised by the antitoxin, and the latter may even have some influence in wresting the toxin from the cells during the early stages of combination. The experiments of Fraser (1896), C. J. Martin (1898), and Heymans (1898) and Dönitz (1899) are interesting in this connection. These observers find that the combination between the toxin and the susceptible cells takes place very rapidly, and that therefore a very short interval only must elapse between the injection of the toxin and the subsequent injection of the antitoxin, if the latter is to be employed to prevent the union. Dönitz, for instance, found that if a single lethal dose of toxin was injected intravenously into a rabbit before the antitoxin, it could be neutralised by the same amount of antitoxin as was required *in vitro*, provided an interval of not more than nine minutes elapsed. If the interval was longer, then a larger dose of antitoxin was necessary to save the life of the animal. If a period of one and a half to two hours, or longer, had passed after the toxin injection, then no amount of antitoxin was capable of averting the death of the animal. The natural interpretation of these results is that the toxin at first circulates free in the blood, during which time the toxin-antitoxin combination takes place just as *in vitro*; later the toxin becomes anchored on to the susceptible cells, but up to a certain period the combination is a loose one, capable of being broken down by the presence of a large quantity of antitoxin; finally the toxin attaches itself so firmly to the cells that no matter how much antitoxin is used the union cannot be dissolved.

The Rate of Disappearance of Antitoxin from the Blood.

The larger the amount of antitoxin injected into an animal the longer, *caeteris paribus*, will antitoxin be capable of demonstration in the blood. The rate of disappearance according to Bomstein (1897) and others is proportional to the concentration of the antitoxin in the blood.

The duration of passive immunity in diphtheria is one of great practical importance and has been studied by a number of observers. Behring (1897) found that there was still a considerable quantity of antitoxin present in the blood of goats 23 days after a large injection, and that antitoxin was demonstrable in the blood of children up to four weeks after a dose of 250 units. This coincides with a wide clinical experience that protection lasts for about four weeks. Passini (1896), after an injection in man, failed to demonstrate antitoxin after 12 days. Bomstein (1898) injected 8000 to 10,000 units into dogs, and failed to find any antitoxin after 18 days. Bulloch (1898) injected 25,000 units into a donkey and traced the antitoxin curve. This reached its maximum in 24 hours. On the fourth day a marked fall was noted, which continued until the 100th day when antitoxin in a measurable quantity was still present. On the 126th day it was below one-tenth of a unit per c.c. Kraus and Joachim (1903) have shown that when diphtheria antitoxin, obtained from the horse, is injected into the rabbit its disappearance is very rapid, a great diminution being discernible even within one hour[1].

Absorption of Antitoxin from the Alimentary Canal.

Ehrlich (1892) clearly demonstrated that suckling animals could absorb antitoxin from the mothers' milk, and this fact has been confirmed by a number of workers. Escherich (1897) investigated the question as to whether antitoxin could be absorbed when administered *per os* or *per rectum.* The experiment was carried out on children of various ages, but the only positive results were obtained when the antitoxin was administered *per os* in sucklings. Rectal injection gave entirely negative results. Since Escherich's work many papers have appeared claiming good clinical results from the administration of antitoxin by the mouth, but experimental results show that there is little or no absorption of the antitoxin obtained from another species. Thus Salge (1904) found that when the antitoxin was an integral part of the milk it could be demonstrated by Marx's (1901) method in the blood of sucklings. When, however, antitoxic serum from the horse was added to the milk, antitoxin could not be found in the blood. Salge concluded that the homologous proteids of the mother's milk could be absorbed by the child, whereas the heterologous proteids of the serum of the horse could not pass through the walls of the alimentary canal. Uffenheimer (1904), who reviewed Salge's paper, said that his own experiments on animals (the antitoxin being measured by Marx's method) gave results in opposition to those of Salge. Dzierzgowski (1899) found that when the antitoxin was introduced into the empty stomach of the rabbit a slight immunity was conferred. Hewlett (1902) could find no trace of absorption of antitoxin from the stomach or rectum in the case of guinea-pigs and rabbits. The factors which hinder diphtheria toxin, introduced into the alimentary canal, from exercising its toxic action are no doubt at work in preventing the absorption of the antitoxin. The hydrochloric acid of the gastric juice probably plays an important part. Oppenheimer states that neutralised pepsin, pancreatin and bile are not very harmful. Pick found that two-thirds of the antitoxin was destroyed during a trypsin digestion experiment lasting nine days. Intestinal bacteria probably exert a destructive influence on the antitoxin. Though colloidal substances with large molecules diffuse with difficulty through an animal membrane, this does not prevent the passage of antitoxin in the suckling. It is possible, however, that the homologous or heterologous nature of the antitoxic serum may be an important factor in influencing absorption.

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[1] This subject is closely associated with the question of the production of anti-antitoxin dealt with in another section, p. 568.

THE QUESTION OF THE PRODUCTION OF ANTI-ANTITOXIN.

Does an antitoxic serum obtained from one species, when injected into another species, give rise to the production of an anti-antitoxin? The rapid disappearance of antitoxin from the blood of passively immunised animals would be readily reconcilable with such an hypothesis.

Pfeiffer and Friedberger (1902 and 1903) were able to demonstrate that an anti-antibody could be obtained to a bacteriolytic serum. The injection into rabbits of a cholera-immune serum from the goat gave rise to the formation of an anti-bacteriolytic serum with "anti-cholera amboceptors."

From this observation it might have been anticipated that the demonstration of an anti-antitoxin would be an easy matter. This, however, is not the case, and a number of observers, among whom may be mentioned Kraus and Eisenberg (1902), and Kraus and Joachim (1903), have reported experiments which show that in the case of diphtheria the injection of a heterologous antitoxic serum into an animal does not give rise to an anti-antitoxin.

Certain observations relating to the production of specific precipitins bear on this subject. Thus Atkinson (1903) showed that the sera of rabbits, some of which had been injected with normal horse serum, others with antitoxic serum, in both cases produced precipitins which were capable of throwing down the substance to which the antitoxin adheres. Sacharof (1905) also found that diphtheria antitoxin in horse serum is attached to the elements which give rise to precipitin formation, and that the precipitate obtained from serum by the use of this precipitin includes the antitoxin adhering to the proteids. That a similar process occurs in the animal body was shown by the following experiment. Into two rabbits of equal weight, of which one was normal and the other had previously received an injection of normal horse serum, the same quantity of diphtheria antitoxin was injected. After a short time the animals were bled and their serum was tested for the presence of antitoxin against a certain dose of toxin. It was found that a larger quantity of serum from the animal which had received the injection of normal horse serum was required in order to neutralise the dose of diphtheria toxin.

It must be noted that these results do not prove the existence of a substance which acts *per se* as an anti-antitoxin; the apparent action in that direction may be merely a secondary effect of the precipitin action.

INHERITED IMMUNITY.

Transmission of acquired immunity to offspring.

Ehrlich (1892) and Ehrlich and Hübner (1894) first successfully employed experimental methods in investigating the transmission of acquired immunity from the parent to the offspring. A series of experiments to investigate the transmission of immunity in the case of abrin, ricin, and robin was carried out in mice. Later, similar investigations were made in regard to immunity to tetanus toxin, and for these experiments mice and guinea-pigs were employed.

In these experiments Ehrlich used the ingenious method of transferring the young of the normal mouse to an immune foster-mother, and the young of an immune mother to a normal foster-mother. The conclusions arrived at were summed up as follows.

1. The idioplasm of the sperma cannot transfer immunity.
2. The ovum is also incapable of transferring immunity.
3. The immunity which can be observed in the progeny at birth originates from maternal antibodies received through the placental

blood. The immunity is of short duration, lasting up to three to four weeks.

4. The milk is the actual agent by which the immune substances are conveyed to the suckling.

5. The longer the maternal milk is supplied the greater is the immunity attained.

6. The immunity is, therefore, passive and is limited to two or three months.

Wernicke (1895) confirmed Ehrlich's results in the case of diphtheria immunity in the guinea-pig.

On the other hand, a number of workers have supported the view that a true, inherited, histogenic immunity can occur. Vaillard (1896), for example, came to the conclusion that the leucocytes of the descendants of animals, which had been immunised against anthrax, inherited a specially active phagocytic power against that bacillus. Tizzoni and Centanni (1893), working with the virus of rabies, and Gley and Charrin (1893), working with *B. pyocyaneus*, have asserted that the immunity can be propagated from the male to the offspring by means of the sperma.

Remlinger (1899) concluded that in regard to the transmission of immunity against the typhoid bacillus the male does not exert any influence, but that the rôle of the female is important, especially when the immunisation has been carried on during gestation; even then the immunity is transient, lasting only one or two months, and is limited to the one gestation after the vaccination. He thought that suckling did not, at least in the case of the rabbit or guinea-pig, play any part in the transmission of immunity.

On the question of the transmission of the agglutinating power there is a mass of literature into which we cannot enter here. In Remlinger's experimental investigations it was found that the male animal played no part, and that it was only when the injections of bacilli were carried out during gestation that the young acquired the property through the placental circulation. The agglutinating property could not be acquired through suckling.

Kraus (1901) was the first to study the transmission of immunity from parents to offspring by the employment of haemolysins obtained by injecting the red blood corpuscles of an animal into an animal of a different species. He came to the conclusion that as no immune substance could be demonstrated in the milk, and that as the serum of the young of the immune rabbits was haemolytic, the haemolysin must pass to the young through the placental circulation. Bulloch (1902), on the other hand, found that immune substance, but no complement, was present in the milk of immunised rabbits. His results entirely confirmed Ehrlich's observations on this subject.

Dzierzgowski (1901) carried out a series of experiments on the inheritance of acquired diphtheria immunity, using hens for his experiments. The immunity was produced by first injecting antitoxin only, and later combined toxin and antitoxin. During the time when serum alone was being used the eggs laid contained no antitoxin, but in those laid after toxin and antitoxin had been injected antitoxin could be demonstrated in the yolk. The antitoxin was found to be associated with a form of globulin, as in the case of the blood serum. The blood of the chicks hatched from some of these eggs contained antitoxin. Dzierzgowski regarded the immunity as of a purely passive character.

THE QUESTION OF ANTI-MICROBIAL DIPHTHERIA SERUM.

From the time of the appearance of Behring's first papers on diphtheria immunity it was recognised that anti-diphtheritic serum had anti-microbic, as well as antitoxic, properties. Behring's observation was confirmed and extended by a number of observers, among whom may be mentioned Klein and Cobbett.

Van de Velde (1897) undertook an investigation to ascertain whether the two properties of the serum could be obtained separately by different methods of immunisation. He immunised five goats by different methods, and the following Table gives a brief outline of the results obtained. In testing the efficacy of the serum in protecting

Goat	Goat treated with	Guinea-pigs treated with serum of goat and afterwards injected with	Result
1.	Strong toxin	1. Toxin	Survived.
		2. Bacilli	Survived.
2.	Washed culture, killed by thymol	1. Toxin	Died in 2½ days.
		2. Bacilli	Survived.
3.	Culture in bouillon; bacilli (killed by thymol) + toxin	1. Toxin	Lived.
		2. Bacilli	Lived.
4.	Weak toxin	1. Toxin	Died in 5 days.
		2. Bacilli	Lived.
5.	Toxin heated to 100° C. for 1 hour.	1. Toxin	Died in 36 hours.
		2. Bacilli	Died in 2½ days.

guinea-pigs he used threefold lethal doses of toxin, or of bacilli free from toxin. The serum was injected into one side of the body and 12 hours later the toxin or bacilli into the other side.

He was thus able to show that the two properties were essentially different, and could be obtained separately according to the method of immunisation employed. He found that in the antitoxin of commerce both the antitoxic and the anti-infectious properties were present. He was unable to separate these by heating the serum to various temperatures.

Lipstein (1903) in re-investigating the question as to whether it is possible to obtain an anti-microbial diphtheria serum indicates that the work of Van de Velde, Klein and others is open to the criticism that their results were perhaps due to the antitoxic, and not to the anti-microbic properties in the serum. He refers to the universal experience that antitoxin is itself capable of protecting against the living organism, and holds that Bandi (1902), who found that various commercial antitoxins did not protect animals against his bacillus, could not have employed a true diphtheria bacillus for his experiments.

In order to neutralise all traces of diphtheria toxin which, as was shown by Kossel, Brieger and Boer, and others, adhere to or are contained in the bacillary bodies Lipstein added diphtheria antitoxin to emulsions of virulent diphtheria bacilli, which had been grown on nutrient agar, and immunised rabbits and guinea-pigs with these mixtures. As the result of a very thorough series of experiments he concluded that in the case of the animals employed by him there was no amboceptor production, and therefore no bactericidal action either *in vitro* or *in corpore*, and that the survival of certain of his
Un the ᵩl animals was due to the traces of antitoxin, sufficient
there is a mass oı ᶠord protection as shown by control experiments.
Remlinger's experimentaıecently been aroused in the question of an
animal played no part, and ɩ.work of L. Martin (1903). By injecting
bacilli were carried out during gεʋ or intraperitoneally into a horse he
property through the placental circuʳglutinating properties (see p. 226).
could not be acquired through sucklii,he property when applied locally
Kraus (1901) was the first to stuc number of living bacilli in the
from parents to offspring by the emplc pain quickly disappears and the
by injecting the red blood corpuscles o detached than when only anti-
a different species. He came to the co
substance could be demonstrated in the n.corporating the dried serum
of the young of the immune rabbits was les. The great value of such

a serum would lie in the reduction by its means of the duration of the period of isolation and residence in hospital, so often necessitated by the persistence of the bacilli in the throats of patients long after recovery from all signs of disease.

Some observations on this subject were made by the Serum Department of the Lister Institute, two horses being injected intravenously first with killed and then with living bacilli. The sera obtained agglutinated the race of bacillus which was employed in dilutions of 1 : 200 and 1 : 300. Clinical tests were made on a series of cases with pastilles containing the serum. No shortening of the period of persistence of the bacilli in the throat was found.

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No 16

ON THE NATURE OF THE FAT CONTAINED IN THE LIVER, KIDNEY, AND HEART. By PERCIVAL HARTLEY, *Grocers' Company Research Scholar.*

(*From the Lister Institute of Preventive Medicine.*)

THERE are many indications that in the active cellular organs of the body the fat exists not so much in the form of simple glycerides of the higher fatty acids as in that of lecithin and similar complex bodies. A more complete knowledge of such complex substances is desirable in order to understand the rôle played by fats in the life of the cell, and something may be learnt from the study of the fatty acids obtained from them by saponification. For this reason Liebermann's method, with slight modifications, has been used.

In the course of experiments which have been carried on for some time by Leathes(1) it has become more and more obvious that the higher fatty acids obtained from the liver or heart or kidneys, behave, in certain respects, differently from those which may be obtained by the same methods from adipose connective tissue. From the organs a larger amount of higher fatty acids soluble in ether but insoluble in petroleum ether is always obtained, and if the acids soluble in petroleum ether are exposed to the air, or still more if they are heated, a part is found to have become insoluble in this solvent. Furthermore, on applying the method proposed by Farnsteiner, for separating acids of the linoleic and linolenic series from acids more saturated, evidence was obtained for the presence in considerable amount of acids less saturated than oleic acid. This seemed to point to the presence, in the fats of the organs examined, of unsaturated acids different from those occurring in the fat stored in adipose connective tissue. It seemed possible that if these differences could be elucidated, some light might be thrown on the nature of the changes which fats undergo when they leave the connective tissues, and enter the stream of metabolic change in the active organs of the body.

With this end in view, I proceeded, at the suggestion of Dr Leathes, to whom I am indebted for much kindly help and advice, to collect the fatty acids liberated from the liver and other organs of different animals, and to examine their properties and attempt the identification of the several acids contained in the mixture. In the present paper I shall confine myself to the results of the examination of certain properties of the acids.

The first property to be examined was the power of absorbing iodine, which, as is familiar, is a measure of the degree to which fatty acids are unsaturated. The iodine value, the weight, that is, of iodine absorbed by 100 parts of the fat, has been found in the case of the fats extracted by ether from the organs to be somewhat higher than that of the fat from the connective tissue. Thus Woltke⁽²⁾ found that the iodine value of the fat from the liver of a normal dog was 83·1, from the heart muscle 84·9, and from the kidney 78·0, whereas the connective tissue fat had an iodine value of 62·8. Leick and Winckler⁽³⁾ obtained the following values: heart muscle fat 83·5, connective tissue fat 59·1 in the case of normal dogs, and in the case of sheep, heart muscle fat 67·7 and connective tissue fat 43·9. Rosenfeld⁽⁴⁾ gives the following figures for the iodine value of the heart muscle fat of four dogs: 53·47, 58·55, 60·4, 41·8. It will be observed that the iodine value of the organ fats is somewhat higher than that of the connective tissue fat. As the iodine value of olein is 85·6, it has been assumed that the higher iodine values obtained for fats derived from the organs indicate that they contain relatively more olein than the connective tissue fat. The experimental results given later in the paper show clearly that this difference between the fat of the various organs and that contained in the connective tissue is not to be explained simply by the presence of varying proportions of olein.

By the method described below, the iodine value for the fatty acids obtained from connective tissue fat, it is true, is of the same order as that found by previous investigators; but the values for the fatty acids from the organ fats are higher than the iodine value of oleic acid, and far higher than any before recorded[1]. The numbers varied from 115 to

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[1] While this work was in progress a paper appeared (Erlandsen, *Zeits. f. physiol. Chem.* LI. p. 71, 1907) in which a substance cuorin, obtained from ox-heart muscle, is described. This substance is a monoamido-diphosphatide and yields on hydrolysis fatty acids, the mean iodine value of which is 130·1. The lecithin from the same source yields fatty acids having a mean iodine value of 110.

In this connection it is interesting to note that Henriques and Hansen examined the fatty acids obtained from the lecithin of the yolk of egg, and from the high iodine values they obtained, argued that a considerable portion of the acids in the lecithin must belong to the linoleic series. (*Skand. Archir f. Physiologie*, XIV. p. 390.)

135. This indicates the presence of fatty acids of the linoleic series ($C_nH_{2n-4}O_2$). The unsaturated acids were separated from the saturated acids and the iodine value of the former determined. This was found to be greater than that of linoleic acid and indicates the presence of acids of the linolenic series ($C_nH_{2n-6}O_2$). An experiment on the bromination of the unsaturated acids not only confirmed this, but also afforded evidence of the presence of an acid or acids containing four unsaturated linkages.

The nature of these fatty acids is being more completely investigated.

Method of preparing the fatty acids. The method which has been most frequently used for the extraction of fat is that of Rosenfeld. This method consists in extracting the dried tissue with alcohol and chloroform and taking up the united extracts with ether. It is well known that this ether extract does not consist wholly of neutral fat. Glikin(5) has examined such ether extracts and has shown that some samples contain 16·12 % lecithin and 3·66 % "ptomaine." Leathes has shown that on saponification with alkalies, the ether extracts yield only 65 % of fatty acids, whereas pure animal fat from the connective tissues yields about 95 % of fatty acids. Since it would be difficult to obtain the fats in a pure form from these ether extracts, it was decided to prepare the fatty acids from the organs by direct saponification. The method adopted has the advantage of simplicity and rapidity, permits of the use of large quantities of materials, and gives a final product consisting of little else but the higher fatty acids.

The method as carried out on the liver will be described in full, but the same procedure has been followed with the heart muscle and the kidney, and the connective tissue fat which has been examined has been taken through every stage of the treatment in precisely the same way.

All visible adherent fat etc. was first carefully removed from the organ, the liver substance cut into small pieces and finally minced in a machine. In most cases the whole of the minced tissue was used for experiment. It was placed in a large flask, and a solution of caustic potash (500 gm. potash and 500 c.c. water) added. 250 c.c. of this solution were used per kilogram of fresh tissue. The mixture was heated on the water-bath and shaken at intervals. When all the tissue had dissolved, methylated spirit was added, and the heating continued, with further addition of spirit, until saponification was complete. The solution was then cooled, diluted somewhat with water and a 10 %

solution of sulphuric acid added until the presence of free mineral acid could be detected in the solution. The acidified solution was heated on the water-bath until the precipitated fatty acids had risen to the surface; the lower layer of clear fluid was then syphoned off. A large volume of water was added, the whole heated on the water-bath until the precipitated acids collected on the surface and the lower layer of wash liquor syphoned off as before. The washing was continued until the wash liquor was no longer acid, and almost colourless. The precipitate was transferred to a filter and left to drain overnight, after which it was dried *in vacuo* over sulphuric acid for 2 or 3 days. When dried as completely as possible the precipitate was extracted in a Soxhlet apparatus with petroleum ether (B.P. 45°—65°) for 6 or 8 hours. The petroleum ether extract was diluted with 2 litres, or more, of the same solvent and allowed to stand for 24 hours. At the end of this time a dark brown substance separated from the solution. This substance is apparently soluble in a concentrated solution of fatty acids in petroleum ether, but insoluble in a more dilute solution. It solidifies to a hard mass. The amount obtained varied in different experiments, and frequently contained nitrogen and sulphur. It has not been further investigated. It was removed from the solution by filtering, and the petroleum ether distilled off from the filtrate on the water-bath, the last traces being removed by heating at reduced pressure in a slow current of dry carbon dioxide. On cooling, the fatty acids solidified to a yellow or light brown mass, consisting of an oily and a crystalline part.

The amount of higher fatty acids obtained from the various organs was determined and is expressed in the following tables in units per cent. of fresh substance used. The iodine value of the mixed higher fatty acids was determined immediately after the petroleum ether, in which they were dissolved, had been distilled off. Wijs' method for determining the iodine value has been used throughout.

The following table contains the results obtained for the higher fatty acids from the various organs of the pig, the goat and the dog.

Source of fatty acids	Weight of fresh substance	Fatty acids %	Iodine value of fatty acids
Pig:			
Liver E	700 gms.	2·86	126·4
Liver F	950	2·58	134·1
Liver G	870	2·44	134·7
Kidney	180	2·37	116·2
Heart Muscle	230	1·75	134·4
Pericardium	—	—	66·1
Connective tissue	—	—	53·9

Source of fatty acids	Weight of fresh substance	Fatty acids %	Iodine value of fatty acids
Goat:			
Liver A	535	2·38	113·5
Liver B	370	3·42	119·6
Kidney (2 pairs)	118	—	120·6
Kidney (7 pairs)	420	2·11	116·3
Heart muscle (2 hearts)	117	1·69	128·2
,, ,, (4 ,,)	195	2·07	107·7
Pericardium	—	—	43·5
Connective tissue A	—	—	35·9
Connective tissue B	—	—	43·8
Dog:			
Liver A	360	2·76	114·6
Liver B	200	2·78	127·6
Kidney B	70	2·71	120·1
Heart muscle B	87	2·20	127·2
Pericardium B	—	—	63·3
Connective tissue B	—	—	60·5

A series of experiments on the higher fatty acids from the human liver and heart muscle has been carried out. The results are given in the following table: an abstract of the post-mortem report is also given:—

Source of fatty acids	Weight of organ	Weight of fresh substance	Fatty acids %	Iodine value of fatty acids
Liver, X.	1380 gms.	1240 gms.	3·98	117·8
Liver, A. P.	450	350	2·59	126·4
Liver, W. H.	1685	800	2·85	123·0
Liver, L. R.	275	245	2·35	128·0
Heart muscle, W. H.	—	102	2·80	120·0
,, ,, H. S.	—	162	2·36	127·9
Omentum	—	—	—	65·0

X. Cause of death: appendicitis and general peritonitis. No further details available.

A. P. Age 15 months. Cause of death: burns, meningitis. Liver pale and soft, but showed no other pathological changes.

W. H. Age 56. Cause of death: intestinal obstruction and lobar pneumonia. Body jaundiced. Peritoneum healthy. Liver pale, soft and greasy. Heart healthy.

L. R. Age 7. Very poorly nourished. Disease: tubercle of lungs, bronchial and mesenteric glands, spleen and liver. Liver showed only a few miliary nodules, principally on the serous surface, otherwise it appeared quite normal.

H. S. Age 54. Cause of death: chronic nephritis, renal calculus, uraemia. Body very fat. Peritoneum healthy. Epicardial fat of the heart very much increased and viscus enlarged. Left ventricle hypertrophied, and other chambers dilated. Moderate atheroma of aortic cusps and great vessels.

The results given in all the above tables differ in a striking manner from those previously obtained, examples of which are given (p. 18): the iodine value of the higher fatty acids from the organs is much higher than the iodine value of the "ether extracts" obtained by other methods. As the iodine value of a neutral fat is somewhat lower than that of the corresponding fatty acid (*e.g.*, the iodine value of olein is 85·6, that of oleic acid 90·0) slightly higher values than those previously obtained were to be expected. But instead of an increase in iodine value of about 5 %, increases of more than 50 % have been obtained in many cases. Experiments have been carried out with the object of determining the cause of this large increase in iodine value. From one portion of a pig's liver, an ether extract was prepared according to the method of Rosenfeld, and this was compared with the higher fatty acids obtained by saponification of another portion of the same liver.

50 grams of fresh liver tissue were dried by heating first on the water-bath, then in an oven at 105°, and finally *in vacuo* at 100° for two days. The whole was finely powdered before the final drying *in vacuo*. The dried substance was boiled for 15 minutes with alcohol, and then extracted for 6 hours with chloroform, boiled again for 15 minutes with alcohol and again extracted for 6 hours with chloroform. The united alcohol and chloroform extracts were taken up in ether, the ether distilled off, the ether extract weighed, and the iodine value determined. The ether extract was then saponified, the fatty acids precipitated with sulphuric acid, washed, dried, and extracted with petroleum ether. The fatty acids were weighed and the iodine value determined.

600 grams of the tissue from the same liver were saponified in the usual way and the iodine value of the higher fatty acids determined. The results are given in the two following tables:

Extraction method.

Fresh tissue	Dry substance	Ether extract (on dry substance)	Iodine value of ether extract	Fatty acids from ether extract	Iodine value of fatty acids
50 gms.	14·27 gms.	18·24 %	78·4	69·4 %	113·3

Saponification method.

Fresh substance	Fatty acids %	Iodine value of fatty acids
600 gms.	3·35	120·0

It will be observed that the iodine value of the "ether extract" (78·4) is of the same order as that obtained under similar conditions by previous observers. If this ether extract consisted of pure neutral fat, on saponification it should yield 95 % fatty acids and the iodine value of these fatty acids should be about 83. Quite different results are, however, obtained by saponification of the ether extract. Only 69·4 % higher fatty acids are obtained, this being in agreement with Leathes(1) and Paton's(6) results, and the iodine value of these fatty acids is 113·3, this figure being slightly lower than that obtained for the fatty acids obtained by direct saponification. It is thus evident that the ether extract contains substances other than neutral fat, and it is to the impurity of the ether extracts that the low figures previously obtained for the iodine value must in part be ascribed.

Changes undergone by the fatty acids on exposure to air. Specimens of fatty acids were left exposed to the air for varying intervals of time and the iodine value determined at the end of that time. The following results were obtained:—

Source of fatty acids	Iodine value of freshly prepared acids	Time exposed to the air	Iodine value after exposure
Pig Liver, B.	123·0	8 weeks	65·0
		14 ,,	60·1
Pig Liver, G.	134·7	2 ,,	116·0
		8 ,,	70·8
Human Liver, W. H.	123·0	1 ,,	113·3

The higher fatty acids from an ox liver, prepared by Leathes, were examined after being exposed to the air for two years. It was found at the end of this time that only about one half of the higher fatty acids dissolved in petroleum ether. The iodine value of this soluble portion was 48·3. The portion insoluble in petroleum ether was resinous in appearance. It dissolved almost completely in ordinary ether. The iodine value of this portion soluble in ordinary ether was 59·4.

The fatty acids from the organs undergo change on exposure to the air, shown by the decrease in iodine value. This change is similar to that undergone by the so-called "drying oils"; these oils or fats are glycerides of the doubly or trebly unsaturated linoleic and linolenic acids. This change occurs more rapidly at higher temperatures.

Further examination of the fatty acids from pig's liver. The results given above indicate that the fatty acids from the liver, the kidney, and the heart muscle, have iodine values of the same order. Whether the acids from the three organs are identical is a matter for further investigation. The following experiments were carried out with fatty acids from pig's liver, as this was the most convenient source from which fairly large quantities could be obtained.

I. *Further purification of the fatty acids.* Cholesterol and unsaponified matter may be present along with the fatty acids extracted by petroleum ether. In order to remove these, if present, the following experiment was performed.

20 grams of fatty acids from pig's liver were dissolved in a 30 °/₀ solution of caustic soda, an excess of alkali being used. The alkaline solution of the soaps was extracted for 56 hours with ether in a continuous extraction apparatus. The alkaline solution was then acidified with 10 °/₀ sulphuric acid, the precipitated acids extracted with petroleum ether,

and the solvent evaporated off on the water-bath. The product obtained was faintly yellow in colour. The iodine value was determined.

Iodine value of fatty acids before the above treatment = 130·0.
Iodine value of fatty acids after the above treatment = 125·3.

Thus, the high figures obtained for the iodine value of the fatty acids in the experiments previously described are not due to substances other than fatty acids.

II. *Determination of mean molecular weight.* The mean molecular weight of the fatty acids obtained as the final product in the above experiment was determined by titration with deci-normal sodium hydrate solution.

0·5238 gm. mixed fatty acids required 18·0 c.c. $\frac{N}{10}$ NaOH.

Mean molecular weight = 291.

0·5684 gm. mixed fatty acids required 19·7 c.c. $\frac{N}{10}$ NaOH.

Mean molecular weight = 288.

The molecular weight of stearic acid is 284.

III. *Separation of the saturated and unsaturated acids.* The separation is based upon the solubility of the lead salts of unsaturated fatty acids in ether.

The fatty acids were dissolved in caustic potash, and the excess of alkali neutralised with acetic acid. A 7 % solution of lead acetate was heated to boiling and gradually added, with constant shaking, to the hot solution of the soaps. On cooling, the clear supernatant fluid was poured off and the lead soaps washed three times with warm water, and then dried. The lead soaps were warmed gently with ether, the ethereal solution filtered off and shaken with 20 % HCl. The precipitated lead chloride and aqueous solution were separated, the ethereal solution washed with water, and the ether evaporated off, the last traces being removed in a stream of dry carbon dioxide. The iodine value of the unsaturated acids was determined.

Source of fatty acids	Iodine value of mixed fatty acids	Iodine value of unsaturated acids
Pig Liver, B.	123·0	189·3
Pig liver, H.	134·0	203·2
Ox liver, A.	111·4	173·8

Since any oleic acid present in the original fatty acids would be contained among the unsaturated acids thus obtained, and since the iodine value of oleic acid is 90 and even that of linoleic acid only 181, it is clear that if the mixed unsaturated acids have an iodine value higher than 181, this must be due to the presence of acids more unsaturated than linoleic acid. Further, the more oleic acid is present in the mixture, the more there must be of these acids more unsaturated than linoleic acid.

IV. *Bromination of the unsaturated acids.* Unsaturated acids readily unite with bromine forming addition products. Dibromo- and tetrabromo-compounds (obtained from acids of the oleic and linoleic series respectively) are easily soluble in the common organic solvents. Hexabromo-compounds (obtained from acids of the linolenic series) are sparingly soluble in the same solvents, and an octo-bromo-addition product has been described ($C_{17}H_{26}O_2Br_8$) which is almost insoluble.

The high iodine values of the unsaturated acids from the liver indicate the presence of fatty acids containing three and possibly four unsaturated linkages, and should give on bromination insoluble addition products.

Two grams of unsaturated acids were dissolved in carbon tetrachloride and the solution immersed in ice. 1 c.c. of bromine was added, drop by drop. The whole was allowed to stand, cooled, in a stoppered flask for three hours. A white solid separated; this was filtered off, repeatedly washed with cold carbon tetrachloride, dried and weighed: weight, 0·76 gm. The substance became very dark-coloured at 240°, but did not melt. To this dried, insoluble addition product 70 c.c. of dry ether were added, shaken repeatedly and the part still insoluble in ether filtered off, dried, and the bromine estimated by Carius' method.

0·2023 gm. substance gave 0·3261 gm. AgBr	Br = 68·5 %
Calculated for $C_{18}H_{30}O_2Br_6$	Br = 63·3 %
Calculated for $C_{18}H_{28}O_2Br_8$	Br = 69·8 %

Thus the presence of fatty acids containing three unsaturated linkages is confirmed, and evidence obtained of the presence of an acid or acids still more unsaturated than these.

CONCLUSIONS.

1. The higher fatty acids from the liver include in addition to saturated acids and acids of the oleic series, considerable quantities of acids of the series $C_nH_{2n-4}O_2$, $C_nH_{2n-6}O_2$, and possibly $C_nH_{2n-8}O_2$.

2. The fatty acids from the kidney and the heart muscle have iodine values of the same order as those of the liver fatty acids, pointing to the presence in these organs, too, of similar acids, in similar amounts.

3. The higher fatty acids from the organs undergo change on exposure to the air. The iodine value diminishes and a great portion of the fatty acids becomes insoluble in petroleum ether.

4. The unsaturated fatty acids yield, on bromination, addition products insoluble in ether, carbon tetrachloride, alcohol, acetic acid etc., similar, therefore, in their properties to the brominated acids of the series $C_nH_{2n-8}O_2$.

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N° 17

ON THE NATURE OF THE FAT CONTAINED IN NORMAL AND PATHOLOGICAL HUMAN LIVERS.[1]

By Percival Hartley and A. Mavrogordato.

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In a paper "On the Nature of the Fat in the Liver, Heart, and Kidney" (1907) it was recently shown by one of us that the fatty acids normally to be obtained from the liver of several species of animals, including man, were to a considerable extent acids of series less saturated than the oleic series, and that the iodine value (the percentage amount of iodine absorbed) of these acids was very much higher (115 to 135) than that of the acids occurring in the simple glycerides of connective-tissue fat (dog, 62; goat, 43; pig, 54; man, 65). This fact had been overlooked previously, owing to the custom of taking the iodine value of an extract which had been obtained from the dried organ by the use of some fat solvent. This had been regarded as the iodine value of the fat in that organ, and as comparable with the iodine value of the fat that can be melted out of the connective tissues.

The method of extraction most commonly used when the iodine value of the fat of the liver or heart has been hitherto determined is the method of Rosenfeld, and Leathes (1904) pointed out that extracts so obtained from the liver of rabbits contain only 60 to 65 per cent. of higher insoluble fatty acids, instead of 95 per cent. as yielded by pure fat from the connective tissues. In the fat obtained from the liver of a pig by Rosenfeld's method the iodine value found was 78·4; but this fat on saponification yielded only 69·4 per cent. of higher fatty acids, and the iodine value of these acids was 113·3.

Furthermore, the unsaturated acids in the fatty compounds occurring in the liver, as was shown in the same paper, readily undergo oxidation when kept exposed to the air, or when heated, and in this way acquire a lower iodine value. The prolonged heating of the powdered tissue with a view to drying, which necessarily precedes the preparation of an extract by a method like that of Rosenfeld, must tend to lower the iodine value. The same pig's liver referred to in the last paragraph, when treated by direct saponification of the fresh tissue, yielded fatty acids with the iodine value 120, whereas the acids obtained from the extract which had gone through the whole Rosenfeld procedure gave this value as 113·3. For these reasons a determination of the iodine value of an extract of dried tissue is of little value, and the figures obtained should not be compared with the iodine value of an extract of the adipose connective tissue, which contains little or no impurity. This is shown by the following figures for the iodine value of the myocardial fat of four normal dogs: 53·47, 58·55, 60·4, 41·8 (Rosenfeld, 1906). The difference between the extremes of these four numbers (18·6) may be due to varying amounts of glycerides of unsaturated acids, or to varying amounts of substances other than fat, or to varying degrees of oxidation which the unsatured acids have undergone. The mean of the four values is less than the iodine value of the fat from the connective tissue of the dog.

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[1] Received December 18, 1907.

Now, the determination of the iodine values of fat from diseased organs has been the foundation of important arguments in pathology, and it is unfortunate that these determinations should have been in so many cases liable to such gross errors. As an example, the experiments of Leick and Winckler (1902) may be quoted. Their experiments are based upon the difference in iodine value of the fat from the connective tissue and the fat from the heart muscle or liver. The iodine values of fat from normal and poisoned dogs and sheep were first determined. A dog was then starved, afterwards fed on mutton fat, and finally poisoned with phosphorus, and the fats examined. The following results were obtained:—

	Subcutaneous Fat.	Myocardial Fat.
Normal Dog	56·1	70·7
Dog, poisoned with Phosphorus . . .	58·6	82·9
Normal Sheep	38·2	58·2
Sheep, poisoned wlth Phosphorus . . .	36·9	64·1
Dog, fed on mutton fat, poisoned . . .	43·3	64·6

Leick and Winckler concluded that a transference of fat from the connective tissues to the heart had occurred. The numbers given, moreover, do not represent the great difference in character between the fats of the heart and the adipose tissue.

It may be, and probably is, true that the argument based and conclusions drawn from such figures as these would be confirmed and established by the determination of the iodine value by less exceptionable methods, and such confirmation is highly desirable.

The present paper contains the results of the first series of determinations of the amount of fat in normal and pathological human fatty livers by the methods indicated below, which we believe to be the best at present available.

METHODS.

The livers were examined as soon after the post-mortem examination as possible. In cases of unavoidable delay the liver was kept in an ice-chest. All visible adherent fat was first removed, and the liver tissue was minced in a machine. Two hundred grms. of the liver pulp were used for the determination of the iodine value of the higher fatty acids, and two portions of fifty grms. each were used for the estimation of the higher fatty acids. Sections were also prepared in most cases for microscopical examination.

DETERMINATION OF HIGHER FATTY ACIDS.

Fifty grms. of liver pulp were dried by heating on the water-bath under alcohol, the whole being repeatedly rubbed with a pestle. As the water and alcohol evaporated a coarse powder was obtained, which was ground to as fine a powder as possible. This was heated for half an hour in an air oven at 105°, and finally heated in vacuo at 80° for six or eight hours. The higher fatty acids were determined in one of two ways (sometimes by both methods), each of which is a slight modification of Liebermann's methods.

First Method.—This was first used by Leathes. A quantity of the dried organ was accurately weighed out and potassium hydrate solution (sp. gr. 1·54) added, and the whole heated on the water-bath for two hours. Fifty c.c. spirit were then added, and the heating continued for one and a half to two hours. The whole was cooled, diluted somewhat with water, and 10 per cent. sulphuric acid added in slight excess. The whole was then heated again, and the precipitated fatty acids collected on a double filter and washed. The precipitate was allowed to drain overnight, and was then dried in vacuo over sulphuric acid. It was finally extracted in a Soxhlet apparatus with petroleum ether (B.P., 45–65) for eight hours, the solvent evaporated off, and the fatty acids weighed.

Second Method.—Five grms. dried substance were weighed out into a specially constructed flask having a long wide neck and a mark indicating a volume of 240 c.c. Thirty c.c. potassium hydrate solution (sp. gr. 1·54) were added, and the mixture heated on an asbestos gauze for half an hour. Thirty c.c. spirit were added, and the heating continued for twenty minutes more. The flask was then cooled and immersed in ice, and 100 c.c. 20 per cent. sulphuric acid (sp. gr. 1.145) were added in small portions. Fifty c.c. petroleum ether were added from a pipette, and the flask closed by a well-fitting rubber stopper. The flask was then shaken vigorously at two-minute intervals for one and a half hours. Saturated common salt solution was added until the liquid in the flask reached the graduation mark, the whole shaken twice more and left to stand overnight. From the clear layer of petroleum ether solution 20 c.c. were withdrawn and transferred to a weighed flask. After evaporation of the ether, the weight of fatty acids was obtained.

As two somewhat different methods had been used during the series of determinations a comparison of the two methods was made. The following results were obtained :—

Case.	Higher Fatty Acids per Cent.	
	Method 1.	Method 2.
Lobar Pneumonia .	13·7	13·6
No. 630 . .	25·3	25·5
Empyema . .	21·5	21·47
Accident . .	17·2	17·3
Carcinoma . .	47·6	46·9

DETERMINATION OF THE IODINE VALUE OF THE HIGHER FATTY ACIDS.

Two hundred grms. of fresh tissue were heated on the water-bath with 50 c.c. potassium hydrate solution (500 grms. KOH, 500 c.c. H_2O) until all the tissue had dissolved. Fifty c.c. spirit were then added, and the heating continued until saponification was complete. The solution was cooled, diluted with water, acidified with 10 per cent. sulphuric acid, and the whole heated on the water-bath until the fatty acids had collected on the surface of the liquid. The lower layer of fluid was siphoned off. The acids were washed repeatedly with hot water, until the washings were acid free and colourless. The acids were finally thrown on to a filter and allowed to drain overnight, and then dried completely *in vacuo* over sulphuric acid. They were extracted in a Soxhlet apparatus with petroleum ether (B.P., 45° to 65°). The petroleum ether solution of the fatty acids thus obtained was poured into a litre of petroleum ether (B.P., 45° to 65°) and allowed to stand overnight, and then filtered, the ether evaporated off on the water-bath, the last traces being removed by heating on the water-bath at reduced pressure in a stream of dry carbon dioxide. The iodine value of the fatty acids was immediately determined, Wijs' method being used.

It is necessary that the above operations should be carried out as quickly as possible, otherwise the figures obtained for the iodine value may be too low in consequence of changes in the unsaturated fatty acids due to oxidation.

The following table contains the results of the examination of a number of normal and pathological livers. The experiments were begun in May 1907. The cases are arranged to show the chief result of our experiments, namely, that as the amount of fatty acids in the liver increases the iodine value of the fatty acids decreases.

No.	Cause of Death.	Higher Fatty Acids per Cent.	Iodine Value of Higher Fatty Acids.
1	Pernicious Anæmia . . .	12·1	116·8
2	Lobar Pneumonia . . .	13·7	116·8
3	Pernicious Anæmia . . .	14·25	116·0
4	Diabetes	14·4	119·6
5	Toxæmic Jaundice . . .	15·6	109·5
6	Accident	17·2	103·5
7	Empyema	21·5	96·0
8	Phthisis	25·4	96·4
9	Broncho-Pneumonia . . .	38·4	84·9
10	Appendicitis	44·0	91·1
11	Carcinoma of Gall-Bladder . .	47·2	77·8
12	Broncho-Pneumonia . . .	54·6	71·8
13	Ulcerative Colitis . . .	60·9	80·3
14	Accident	66·3	63·0
15	Dysentery	73·5	69·1

EXTRACTS FROM POST-MORTEM REPORTS.

CASE 1.—*Pernicious Anæmia.*—Age, 36. Body pale but fat, and fairly well nourished. Weight of liver, 52½ oz. Acute necrosis microscopically.

CASE 2.—*Lobar Pneumonia.*—Age, $1\frac{11}{12}$. Liver normal.

CASE 3.—*Pernicious Anæmia.*—Age, 50. Weight of liver, $65\frac{1}{4}$ oz. Liver large, pale, and friable. Body fat diminished. Acute necrosis microscopically.

CASE 4.—*Diabetes.*—Acute onset. Acidosis on admission. Weight of liver, 55 oz.

CASE 5.—*Toxæmic Jaundice.*—Age, $1\frac{10}{12}$. Body jaundiced; microscopically areas of necrosis; diffuse fatty changes.

CASE 6.—*Accident.*—Adult, female. Liver dark brown, friable, and soft. Weight 45 oz.

CASE 7.—*Broncho-Pneumonia.*—Empyema. Age, $2\frac{1}{2}$.

CASE 8.—*Phthisis: Acute Caseous Tubercle.*—Age, 40.

CASE 9.—*Retropharyngeal Abscess; Septic Broncho-Pneumonia.*—Age, 3 months.

CASE 10.—*Appendicitis.*—Age, 10. Weight of liver, $37\frac{1}{4}$ oz.

CASE 11.—*Carcinoma of Gall-Bladder.*—Age, 46. Secondary growths in liver.

CASE 12.—*Broncho-Pneumonia.*—Age, $\frac{11}{12}$. Child had some diarrhœa.

CASE 13.—*Chronic Ulcerative Colitis.*—Liver, $71\frac{1}{2}$ oz. Body extremely emaciated.

CASE 14.—*Accident.*—Liver very fatty.

CASE 15.—*Dysentery.*—Bright yellow fatty liver. Age, 12 months.

DISCUSSION OF RESULTS.

The first point that stands out clearly from the above table is that, in the cases examined, with increasing amounts of fat in the liver we find diminishing iodine values. Now the iodine value of the higher fatty acids from adipose connective tissue, as was shown in an earlier paper (1907), is considerably lower than that of the acids obtained from normal livers. The average value for the acids from adipose connective tissue in man is about 65, while for the acids from normal livers it ranges from 115 to 120. The same kind of differences are found in the pig, dog, and goat. The average amount of insoluble fatty acids to be obtained from human livers is, as the figures in the above table and others we have obtained which are not included in the above table (12·6 per cent., 12·7 per cent., 12·6 per cent., 14·6 per cent., 16·8 per cent.) show, from 12 per cent. to 16 per cent., reckoned on dry weight of tissue. Therefore, so far as the cases included in the above table go, we may make the general statement that, *when the amount of fat in the liver is greater than is commonly found in health, the iodine value of the acids contained in this fat approximates to that of the acids in adipose connective tissue.*

The microscopical examination of sections from the livers examined so far has in every case in which the amount of fat was excessive shown appearances characteristic of "fatty infiltration"; that is to say, the fat is collected in the cells into definite drops large enough in the more extreme cases to fill up the liver cell. Necrotic changes in the cells have been found only in cases in which the amount of fat was not outside the normal variations. So far, then, as our examination has gone at present *we have no evidence that "degeneration" is* *accompanied by the change in the character of the fatty acids present, which is indicated by an abnormally low iodine value.* And at the same time, *we have no case in which the amount of fat is above the normal while the iodine value of the acids is maintained at the normal figure, i.e.* 115 *to* 120.

Such cases we are still searching for, and if we find them they will constitute groups of a different type from those abnormal cases that we are at present concerned with, and will require a different explanation. The cases dealt with in this paper are apparently, at any rate, the commonest form of fatty disease of the liver. They present a picture of typical fatty infiltration in which the cells are more or less charged with fat approximating, in the character of the fatty acids entering into its composition, to the fat of adipose tissue, and in proportion to the degree to which they are thus overcharged. In Case 15, for instance, where the amount of fat was the largest, the iodine value of the acids was very low, and only slightly different from that of the acids in adipose tissue. In Case 14, with slightly less fat, the iodine value is still lower, and practically identical with the value for adipose tissue fatty acids.

Such a condition can be supposed to be brought about in one of two ways. In the first place, we may suppose that the excess of fat has been imported from the connective-tissue depôts, and has a low iodine value, because much of it has not been altered in character since it reached the liver. Such a hypothesis would imply, as a further hypothesis, that the liver normally receives fat from the connective tissues whenever that fat is put into circulation for use, and that the changes which food fat is found normally to have undergone by the time we can examine it in the active cellular organs, such as the heart, kidney, or liver itself, changes indicated by the higher iodine valve, are carried out to a great extent, perhaps even exclusively, in the liver, the altered fatty acids in some combination being the material needed by the working cells, and prepared for them from the stored fat of the adipose tissue, in great part by the liver.

On this hypothesis the pathological condition found in the class of cases we have examined would seem to consist in the failure of the liver to work up the fat at a pace commensurate with the rate at which the fat is called up from the storage places.

The main support for such a hypothesis would, of course, be, in the first place, the fact that there is no organ in the body in which precisely the condition described is found, so commonly or in anything like so marked a degree, except the liver; and secondly, the fact that there is evidence that the minor degrees of this condition are physiological, and even that the extreme degrees may be merely temporary. Rosenfeld has described, as a constant result of administering phlorrhizine to starving dogs under certain conditions, a great accumulation of fat of adipose tissue type in the liver—up to 70 per cent. of the dry tissue—and at the same time notes that, on stopping the phlorrhizine and allowing the animals normal food the condition passes off completely.

On the other hand, an alternative explanation may be that the fat of low iodine value found in such large quantity in the liver is made in the liver from carbohydrates. It is known that animals fattened on meal have a firmer fat than those fattened on cake: the former make fat from carbohydrates, and this fat is comparatively rich in solid, that is, saturated fatty acids. And there is some evidence that the synthesis of fat from carbohydrates is especially carried out in the liver.

On this hypothesis the disorder would consist in exaggerated activity of a normal function of the liver.

The experimental data that we have brought forward, while they indicate the possibility of these two alternative hypotheses, do not, of course, make it possible to decide between them.

For the material that we have used we have to thank, in part, the physicians of St. Thomas's Hospital, who allowed us to make use of it; Dr. Boycott, who sent us several of the livers included in our table from Guy's Hospital; and Dr. Ledingham, who procured us material from cases at the Victoria Hospital for Children. Dr. Meek also has kindly supplied us with some of the sections, and allowed the rest to be prepared in his laboratory. Finally, we desire to thank Dr. Leathes for much valuable help and advice during the course of the work.

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Nr 19

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ACIDOSIS IN PREGNANCY.

By J. B. LEATHES.

THE subject which I have been asked to bring up for consideration is one that has as yet not been fully worked out, but the work that has been done has brought to light facts and raised questions that may be of the greatest importance in some of the graver disorders of pregnancy. There are three papers or groups of papers in which, in Germany and America, attention has been called to the significance of the high proportion of the excreted nitrogen that may take the form of ammonia salts in the urine during pregnancy. The first of these is the work of Zweifel [20]. He found in eighteen successive cases of eclampsia that the nitrogen excreted as urea formed a smaller proportion of the total nitrogen than is the case under normal conditions (from 27 to 70 per cent., instead of about 87 per cent.), while the ammonia nitrogen was high (in one case as high as 16·5 per cent., the normal quantity on a normal diet amounting to not more than 5 per cent. and commonly even less than 4 per cent.).

Zweifel has interpreted these figures in ways that are not altogether in harmony with current views, and more particularly ascribes the high percentage of ammonia to the excretion of lactic acid, which he regards as the cause of the eclampsia. He finds lactic acid commonly in eclampsia both in the urine and in the blood. In three cases he found more in the placental blood and in three cases more in the fœtal blood from the umbilical cord than in the maternal blood. On these grounds he thinks the acid is produced in the child and causes poisoning of the mother and is not produced in the mother as a result, for instance, of the fits. Again, in four cases the lactic acid was found in the urine before the fits, and similarly in two cases in the blood. The figures obtained in one of his cases are as follow:—

	Weighing	Lactic acid in milligrammes	Per mille
Placenta	386	181·0	0·7 of blood reckoned as two-thirds the weight of the placenta
Blood of umbilical cord	95	24·5	0·26
Maternal blood	390	32·5	0·08
Urine	820	415·0	0·5

Now lactic acid has been repeatedly found in the urine of epileptic patients. Inouye and Saiki [11] examined the urine passed one or two hours after epileptic fits on several occasions in each of seven epileptic subjects and found lactic acid every time, and in much higher concentrations than Zweifel, up to 1 per cent., or twenty times the amount in the case quoted above. In the intervals between the fits lactic acid could not be found, so that the conclusion drawn is not that the acid is the cause of epilepsy, but that it is produced in the muscles as a consequence of the impeded respiration. There is much evidence that this is the consequence of deficiency in the supply of oxygen to the muscles. After removal from the body the muscles, it is well known, produce lactic acid, but this is not the case if a free oxygen supply is maintained; and if lactic acid has been formed the excised muscles, when supplied with oxygen, are able to cause its disappearance [10]; or if in a living animal the circulation through the hind limbs is interfered with, the venous blood from those parts is found to be charged with lactic acid. It is true that normally the urine contains no detectable amount of lactic acid, and normal blood at most a trace; but 0·3 per cent. was found in a case of normal but protracted labour [3]. It is also necessary to remember that the estimation of lactic acid in urine and blood is very imperfect. If lactic acid in known amount be added to blood and the amount estimated the result is, it may be, 50 per cent. too low, and with urine the results are even less satisfactory still [3], so that it is not possible to attach very great significance to differences between the amounts found in blood from different sources when the amounts are so small. And even granted that the amount in the fœtal blood is sometimes larger than that in the maternal, this is no less than might be expected from the interference with the fœtal circulation liable to occur during delivery.

But in any case lactic acid is a substance that it is difficult to look on as likely to cause profound intoxication. The injection of sodium lactate into the circulation of dogs in amounts far greater than any ever found by Zweifel causes no signs of disturbance; his highest figure is 0·11 per cent., and in the experiments referred to the amount injected was sufficient to give a concentration of 1 per cent., or even 1·5 per cent. in the blood [4] [5].

But, whether the lactic acid is significant or not, the high percentage of ammonia in the urine observed by Zweifel is remarkable, and in some of the disorders of pregnancy considerably higher percentages still have been observed. Thus Williams [19] reports on three remarkable cases of severe vomiting in pregnancy in which the ammonia excreted in the urine accounted for 32, 35 and 45 per cent. respectively of the total nitrogen. Williams found on examining the liver of a case which was clinically precisely similar to these, central necrosis of the lobules resembling that found typically in acute yellow atrophy; and he refers to another case clinically and histologically identical with this one. He therefore regards these as cases of toxæmic vomiting allied to yellow atrophy, and this latter as a fulminating variety of the same disorder. Other cases of severe vomiting in pregnancy in which the ammonia coefficient is normal may be recognised by that sign as of a comparatively innocent type—cases of neurotic or, it may be, reflex vomiting. But when he says this coefficient is increased to 10 per cent. or 15 per cent. it would seem that the diagnosis of toxæmic vomiting is justified and an urgent indication given for the prompt termination of the pregnancy. The following is a brief abstract of his three cases of toxæmic vomiting, with the total nitrogen excretion of twenty-four hours and the percentage ratio of the ammonia nitrogen in the several specimens examined.

TABLE I.—WILLIAMS'S CASES.

Case 1. Vomiting began in second month; became incessant in third month; lost 20 lb. in weight. Pulse 132. Liver dulness 1½ in. above costal margin. No albumin nor casts.

	T. N. g.	N. as NH_3 per cent. of T. N.
Urine for twenty-four hours before abortion ...	4·5 ...	32·0 ×
,, one month after	12·7 ..	9·6

Table I.—Williams's Cases—*(continued)*.

Case 2. Vomiting and indigestion at beginning of second month; incessant for three weeks in third month; lost 15 lb. to 20 lb. Liver dulness normal. No albumin nor casts.

	T. N. g.	N. as NH_3 per cent. of T. N.
In twenty-four hours urine	6·0	16·0
	7·5	20·7
	5·5	35·5 ×
	8·0	32·0
Abortion	6·4	24·0
	9·5	18·0
	8·7	19·0
Vomiting returned fifth day	7·0	25·5
	5·5	21·0
	4·7	12·0
Went out tenth day against advice	4·5	19·0
One month later	—	4·0

Case 3. Vomiting incessant through second month. Liver dulness normal. No albumin nor casts. No jaundice. Post-mortem not obtained; but a similar case showed central necrosis of liver lobules as in acute yellow atrophy.

	T. N. g.	N. as NH_3 per cent. of T. N.
In twenty-four hours urine	7·5	15·8
	9·7	15·3
Abortion	16·8	19·0
	9·0	31·0 45·5 × 24·0 36·0

Death forty-eight hours after operation.

Much the fullest and most valuable set of analyses of the urine in pregnancy is that given by Ewing and Wolf [6]. In addition to the determination of the total amount of nitrogen in the urine, and the amount of that which was present in the form of ammonia, they estimated also the urea, uric acid and creatinine, and consequently were able by subtraction to form an idea of the amount of nitrogen not accounted for by any of these substances. Exact data have been obtained by Folin in precisely this way for a number of normal individuals taking a diet of milk and eggs containing 19 g. of nitrogen. These average normal figures are given for comparison in the first line of the table that follows, which is an abbreviated version of those given in Ewing and Wolf's paper.

Their cases are divided by them into six groups. The first two groups exhibited no abnormal symptoms, but while the urine from Cases 1 and 2 gave normal or almost normal figures, the second group, Cases 3 to 6, showed signs of the abnormal distribution of the excreted nitrogen, which is, in varying degrees, characteristic of the remaining groups.

The next six cases, group C, were clinically alike in that they were all characterised by severe vomiting lasting for a considerable time in the early months of gestation. In the urine of all of these the urea nitrogen forms a low percentage of the total nitrogen. In two-thirds the ammonia is high, in one case very high; but in all six the undetermined nitrogen is uniformly high even in the two cases with normal ammonia coefficients.

The fourth group of cases were clinically different from these; although vomiting was commonly a pronounced feature in them, too, it came on for the most part later in pregnancy, and was accompanied by albuminuria and other signs of renal failure, high tension pulse, indigestion, temporary blindness, headache, or œdema. These cases of pre-eclamptic toxæmia gave somewhat different figures. The ammonia coefficient was comparatively low, if not normal, and only on one occasion in one of the cases reached double figures. Nevertheless the amount of urea was too small, and for the reason that the undetermined nitrogen was in excess. In two-thirds of the cases more than 20 per cent. of the nitrogen of the urine occurred in compounds of undetermined nature on some of the days for which the data are given.

The fifth group is composed of eight cases of eclampsia, of which five recovered and three were fatal. Here, too, the tables show that the output of ammonia was frequently not excessive—in half the cases it did not reach double figures. And yet though the undetermined nitrogen was higher than normal it formed more than 20 per cent of the total nitrogen in only one of the cases, which case recovered. In another case that died the figure would certainly have been high had the analyses been complete. The last group of four cases of acute yellow atrophy showed similarly irregular figures for the distribution of the excreted nitrogen. But the cases in both these two groups were not sufficiently long under observation for the analyses to have the value which attaches to those carried out on the cases in the third and fourth groups.

Table II.—Ewing and Wolf.

	Total Nitrogen g.	Urea Nitrogen percentage of T. N.	NH_3 Nitrogen percentage of T. N.	Undetermined Nitrogen percentage of T.N.	
Normal values (Folin)	19	85–88	3–5	4–6	
A.—Normal values in pregnancy (E. & W.):					
Case 1	4–9·7	74–84	3·5-5·8	8–14·8	
,, 2	—	71–84	2·8-5·8	7–14	
B.—Abnormal values with no symptoms:					
Case 3	6–7·4	63–67	6·2-8·6	15	
,, 4	8	54	11	24	Severe constipation.
,, 5	4–9	67–73	5·2-7·6	13-18·5	
,, 6	7·5–9	70–76	4–7	11–16	Fourth pregnancy; eclampsia with first pregnancy; casts at end of second.
C.—Toxæmic vomiting:					
Case 7	12·5	67–71	14·2-15	13	Persistent vomiting for two weeks; acetone.
,, 8	6·2	72	12	14	Persistent vomiting for four weeks, second and third month.
	—	80	5	16	Four days after abortion.
,, 9	6·5	60	10	18	Persistent vomiting for eight weeks.
,, 10	4·8	67	2·6	22	Persistent vomiting many weeks; jaundice; no acetone; constipation; "neurotic."
,, 11	3·3	47	*21*	24	Persistent vomiting; amaurosis.
Four weeks later	—	18	*43*	26	Acetone in breath; starvation.
	2·2	32	*38*	28	Labour induced (twins).
Two weeks later	—	77	5·5	13	Well; nursing.
Case 12					
Two days before	4·3	79	2·3	13	Vomiting from conception; abortion at three months; death twenty-five days later.
Seven days after abortion ...	2·8	70·5	7	18	
D.—Pre-eclamptic toxæmia:					
Case 13	—	65	6·7	28	Second pregnancy; high tension pulse; indigestion.
	—	56	8·2	16	Abortion, seventh month.
	5·5	74	7	10	Third pregnancy, sixth month.
	6·3	75	4	16	Neuralgia, high tension.
,, 14	9·6	60	10	27	Jaundice; vomiting; high tension pulse.
Five weeks later	—	74	6	14	Condition improved; labour normal four months later.
Case 15	8	62	9	19	Œdema; headache; indigestion; pulse > 100.
,, 16	9·6	65	4	25	Vomiting; high tension; jaundice.
,, 17	5	80	5	10	Acute nephritis.
,, 18	8	68	4	21	Vomiting; high tension; jaundice.
,, 19	16	73	4·4	17	Vomiting; high tension; jaundice; headache.
	2·7	62	5	28	
,, 20	—	73	6	16	Marked œdema; urticaria.
,, 21		58	5·8	33	Headache; amaurosis; œdema; vomiting.

TABLE II.—EWING AND WOLF—*(continued).*

	Total Nitrogen g.	Urea Nitrogen percentage of T.N.	NH_3 Nitrogen percentage of T.N.	Undetermined Nitrogen percentage of T.N.	
E.—Eclampsia :					
Case 22	8·7	70	5·3	19	Five convulsions ; coma ; no acetone.
,, 23	18	67	7	22	Six convulsions ; coma ; no acetone.
	11	78	8	11	Delivered.
,, 24	4·7	73	9	11	Five convulsions.
,, 25	—	42	18	—	Twenty-five convulsions ; coma.
	9·9	79	8	8·5	Delivered.
,, 26	3	68	8·7	15	Delirium ; jaundice ; very high tension pulse ; *no convulsions.*
	8·6	80	7·5	10	Day after delivery.
,, 27	—	36	6	—	Convulsions ; coma.
Next day ...	—	43	14	—	Died ; liver, early thrombosis and autolysis.
Case 28	1·7	62	10	14·5	No acetone ; one fit ; coma ; collapse ; death.
,, 29	4·5	65	14	13·6	Thirty convulsions ; liver, early thrombosis and autolysis.
F.—Acute yellow atrophy :					
Case 30	11	58	4·5	[19][1]	Vomiting to fifth month ; amaurosis ; œdema ; stupor ; convulsion ; coma till death on thirteenth day ; liver extensively necrosed.
Three days later	8	68	17	[13]	
Case 31	—	26	16	[22]	Coma and collapse in sixth month ; liver autolysis ; no necrosis.
,, 32	7·2	64	9·3	[12·5]	Nursing for fourteen months ; no liver dulness.
,, 33	7·2	79	10	[10]	Vomiting one week ; jaundice ; delivery, followed by coma and death in forty-eight hours ; liver necrosed.

[1] Two days after delivery.

It is in the very full and often extended observations of the urine in the third and fourth of the above groups of cases that the great interest of this work lies, for though the cases with one exception were not actually fatal they were obviously serious, and in some instances would no doubt have ended fatally if the pregnancy had not been terminated.

The two types of abnormality are fairly distinct. One is characterised by severe vomiting in the early months with high ammonia coefficients and generally large amounts of undetermined nitrogenous substances, but no renal affection. The cases described by Williams emphasise the gravity of this condition, described as toxæmic vomiting, and with their very high ammonia coefficients give further significance to this abnormality of the urine. The other is an affection of the later months, in which the vomiting is secondary to renal complications, a pre-eclamptic condition in which the urine does not commonly have a particularly high ammonia coefficient, though the undetermined nitrogen appears to be very abundant.

The question raised is: How far are these abnormalities in the urine diagnostic indications of the gravity of the disorder? Do they lie at the root of the malady, as early fundamental symptoms of a disturbance of metabolism, of which, it may be, yellow atrophy or conditions of that order of gravity on the one hand, and eclampsia on the other, may be merely the final climax? If that is so, of course the importance of exact analysis of the urine in pregnancy is obvious, and Williams may be right in suggesting that an ammonia coefficient of 10 per cent. or more should perhaps be taken as an indication for the termination of the pregnancy.

High ammonia coefficients are well known to occur in a number of conditions. In the following table (III.) are some figures obtained by Folin [8] from normal individuals, in the first line on a diet of eggs and milk, rich in nitrogen; in the next four lines on a diet of arrowroot and cream, poor in nitrogen but otherwise sufficient. In these latter the figures given are the lowest observed in each subject for the urea and the highest for ammonia and undetermined nitrogen, the diet having been taken for five days or more in each case. The ammonia is high and also the undetermined nitrogen. It is true the absolute amount of both these forms of excretory nitrogen is smaller than on the full nitrogen diet: 12 per cent. of 3·5 g. = 0·42, while 5 per cent. of 16 g. = 0·8. But then the total nitrogen excretion of most of the patients examined by Ewing

TABLE III.—FOLIN.

	Total Nitrogen g.	Urea Nitrogen percentage of T.N.	NH_3 Nitrogen percentage of T.N.	Undetermined Nitrogen percentage of T.N.
Diet I.—Eggs and Milk N. = 19 g. Average excretion of several subjects	16·0	85—88	3—5	3—6
Diet II.—Starch and cream	3·8	60	13·6	12·8
N. = 1 g.	3·6	62	11·3	10·9
Four subjects	2·7	61	11·4	11·6
Maxima or minima	3·5	59	9·4	15·4

and Wolf was also very low. In Case 11, when the ammonia coefficient was 21 per cent., the total nitrogen amounted to only 3·3 g., so that the total NH_3 was 0·69. And in these cases, besides a low intake of proteid, the supply of food in other forms was, owing to the vomiting, also low, which was not the case in Folin's subjects on the starch and cream diet. Now starvation is another of the conditions in which not only high coefficients but large absolute amounts of ammonia are observed. This is illustrated by the figures given in Table IV., taken from the results of the examination of a professional faster by Cathcart [1], and the figures given by von Noorden [17], from a patient treated for gastric ulcer by complete starvation, are similar (Table V.). In one case by Nebelthau of starvation in hysteria the ammonia coefficient reached the figure of 66 per cent. of the total nitrogen.

TABLE IV.—STARVATION (CATHCART).

	Total Nitrogen	Urea Nitrogen percentage of T. N.	NH_3 Nitrogen percentage of T. N.	Undetermined Nitrogen percentage of T. N.	Absolute Amount of Nitrogen as NH_3
Average of four days on Diet I. (Milk and eggs)	16·0	87	3·5	5·7	0·6
Fast—Fourth day	13·7	82	9·5	3·3	1·3
Sixth day	10·8	78	12·6	3·3	1·35
Eighth day	9·5	71	14·9	6·8	1·4
Fourteenth day	7·8	77	9·5	4·6	0·7

TABLE V.—STARVATION: VON NOORDEN'S PATIENT (GASTRIC ULCER).

Day	T. N.	NH_3	Percentage
1st	10·2	0·8	8
2nd	11·8	0·9	8
3rd	9·9	1·4	14
4th	8·6	1·5	18

That vomiting and diarrhœa in children cause high percentages of NH_3 has been pointed out especially by Czerny and Keller, who found commonly figures varying from 30 to 52 per cent.; and it is well known, too, that it is not necessary that starvation should be complete in order to bring on the condition in which a large proportion of the nitrogen is excreted as ammonia. The abstention from carbohydrate food has the same effect, and it is possible that diabetes, itself the most familiar of all the conditions in which high ammonia coefficients (up to 40 per cent. and more) are common, is in this respect merely a special case of this defect; what carbohydrate is admitted into the body may in diabetes be excreted without combustion, and the same result is attained as if none were taken at all.

In all these conditions the excess of ammonia appears in the urine because the acids produced in metabolism, failing to find fixed alkalies to neutralise them, are neutralised by ammonia that would otherwise have been converted into urea. The acids may either be derived from the oxidation of sulphur or phosphorus set free from broken-down proteid, or may be organic acids that in disordered metabolism—in diabetes, for instance—or in deprivation of carbohydrate food, with or without complete starvation, have to be excreted because they are not oxidised to carbonic acid. Carbonic acid can be removed without loss of bases, whereas the unburnt organic acids must be excreted as neutral salts, and so carry out with them fixed alkali; and it must be remembered that acidosis, if by that we mean merely the excretion of excessive amounts of ammonia that would otherwise be converted into urea, may be due to a shortage of fixed bases as much as to an excess of acids. The salts of organic acids in ordinary diets supply the fixed bases required for removal of sulphuric and phosphoric acids as well as any organic acids that may escape combustion, and a diet poor in such salts must lead to an output of ammonia that would otherwise have appeared as urea. This is probably the explanation, at least in part, of the high NH_3 coefficients in Folin's subjects on a diet which, though plentiful, consisted only of starch and cream (Table III. *supra*). Similarly, too, in an experiment of Landergren's [12] a diet was taken for five days consisting of butter, 250 g.; oil, 190 g.; wine, 170 g. Table VI. gives

TABLE VI.—Landergren.

Diet consisting of butter, oil and wine only.

Day	Total nitrogen	Urea N. g.	NH_3 N g.	= percentage of T. N.
1st	7·1	5·5	0·4	6
2nd	8·5	6·9	0·8	10
3rd	8·8	6·4	1·4	16
4th	7·1	4·2	1·9	25
5th	5·7	2·7	2·1	37

he figures obtained. The high ammonia coefficient on the fifth day is ue not only to the excretion of β oxybutyric acid resulting from the privation of carbohydrate food; another contributing factor was the very small intake of fixed alkali, which could have been present in this diet only as salts of organic acids in the wine.

This same factor, deficiency of alkali, comes into play in some degree in prolonged vomiting, severe diarrhœa (when the stools may be actually alkaline), and in diabetes even, in which the loss of magnesium and calcium due to the prolonged drainage of bases by the unburnt organic acids, especially β oxybutyric acid, has been frequently noted. It may even be the case that the pathology of acidosis turns upon this drainage of bases from proteids and substances of a similar complexity, the needs of which for fixed bases in the proper execution of their functions are not to be measured by the comparatively slight acid character stamped upon them. They are commonly also basic in character as well, and in the absence of fixed alkali may be hampered in their activities by having to play the base to each other's acid.

Another possible significance that may be attached to an increase of the ammonia in the urine is associated with the function of the liver in converting ammonia and carbonic acid into urea. The effect of removing the liver in birds is to cause two-thirds of the nitrogen to be excreted as ammonia, and in dogs Nencki and Pavloff found that after Eck's operation the ammonia in the urine rose till it formed 20 per cent. of the total nitrogen contained in it. The association of acute yellow atrophy with pregnancy, and of the lesions in the liver characteristic of that disease with the cases of toxæmic vomiting described by Williams, have been sometimes regarded as indications for attributing the abnormalities of the urine in pregnancy to disturbance of hepatic functions. But there is less justification for supposing that disease of the liver is likely to lead to a diminished formation of urea than is often admitted. In severe cirrhosis of the liver Weintraud found normal figures for the ammonia coefficient commonly in all but the final stage, and though cases are on record where higher values have been found (Hallervorden, 1·4 to 2·5 g. per diem; Mörner and Sjöqvist, 2·4 g. per diem = 9·5 per cent.; Fawitzki, five cases, ranging from 7·6 per cent. to 17·5 per cent.; Schubert, one case, always more than 10 per cent. and once 17·5 per cent.), nevertheless Weintraud found that even when the coefficient was high the administration of ammonium salts led to an increased output of urea and not of ammonia, just as is normal. This, therefore, points to the excess of ammonia in the urine being due not to failure of the liver or other organs to convert it into urea, but to the presence of unburnt acids in the urine. So, too, in yellow atrophy itself, though, as in Ewing and Wolf's cases, there is generally an abnormal amount of ammonia, only one case is recorded, so far as I have discovered, where it amounted to more than 20 per cent. of the total nitrogen (Münzer 37 per cent). But Münzer, who studied the urine of a case of phosphorus poisoning in detail for several days, and found ammonia coefficients varying from 8 to 18 per cent., was able, by administering on two successive days 6 g. of sodium bicarbonate, to reduce the output on the first day from 16·6 per cent. to 11 per cent., and on the second to 6·2 per cent. This certainly seems to point to the high figures here, too, being due not to a defective formation of urea but to the excretion of acids that should have been converted into carbonic acid.

So far, therefore, as the ammonia figures in the disorders of pregnancy are concerned, before it can be safely maintained that these high figures are a sign in themselves of a toxæmia that is likely to prove fatal unless the most active measures be taken, it is necessary to prove that they are not sufficiently accounted for by some of the attendant circumstances of the patient's condition: the low nitrogen content of the absorbed food, the imperfect nutrition due to the incessant vomiting, the loss of alkali in the vomit, aggravated possibly by the requirements of the fœtus. The facts that the high figures may occur without any symptom of general disturbance (Ewing and Wolf's cases, 3 to 6), and that there may be profound disturbance with normal figures (*ibid.*, Cases 10 and 12, the latter fatal), are difficult to reconcile with such a view. In some of the recorded cases acetone is mentioned as having been detected, which is no more than could be expected, and, therefore, the acids associated with acetone were probably present in sufficient quantity to account for some of the excess of ammonia. The valuable data contained in Ewing and Wolf's paper, and the stray observations outside it, point to the necessity of decisive experimental work before their pathological significance can be positively defined.

The other abnormality of the urine, the large output of unknown substances containing nitrogen, is more striking and more suggestive, for this does not appear among the results of mere starvation, as the figures given by Cathcart (Table IV.) show. The only substances that have been detected giving rise to variations in the nitrogen in this category are amido acids, and though, of course, this does not warrant a prejudice as to the meaning of the figures in disorders of pregnancy, if the pressure of amido acids in abnormal amounts were proved, it is possible that some of the abnormal ammonia figures might be explained in that way, and that the tendency to associate the abnormality of the urine with disturbance of hepatic functions, and to regard it with suspicion as a sign of danger from that quarter, would be strengthened. It is to be remembered that Ewing and Wolf's first six cases, in which no symptoms were present, all showed high figures in this column, at any rate occasionally, so that it is not clear that great importance should be attached to these facts. On the other hand, it may be noticed that all the cases in which there is a note of jaundice occurring were cases in which particularly high figures under this heading were observed.

But it must be confessed that up to the present time such complete analyses as those given by Ewing and Wolf have been carried out for very few, if any, other pathological conditions, and till more is known of the kind of figures which other diseases present in this respect, and more than that, till the nature of the substances referred to as undetermined has been defined, it is hardly legitimate to speculate upon, much less to draw inferences from, variations in the amount of unknown substances, which may or may not be the same in any two cases. The only admissible justification for attempts to interpret this phenomenon will be definite work on the subject.

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The Inhibitory Action upon Subsequent Phagocytosis, exerted on Active Normal Serum by Inactive Normal Serum through which Bacilli have been passed.

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(Communicated by Dr. C. J. Martin, F.R.S. Received April 30,—Read June 13, 1907.)

Considerable difference of opinion still prevails regarding the nature of the opsonic substances present in normal serum. Wright, Bulloch and Atkin, etc., uphold the view that the opsonin of normal serum is a simple thermolabile body. Muir, on the other hand, regards the opsonin as a body which behaves like complement, while Dean holds that it is essentially thermostable and in all probability co-operates in its action with a thermolabile complement. The demonstration of anti-bodies by complement-deviation experiments (Bordet, Gengou, Pfeiffer and Friedberger, etc.) has recently proved fruitful in connection with the bacteriolysins, hæmolysins, precipitins of immune sera, and the following experiments were designed to test whether, by a similar method applied to phagocytosis, the presence in normal serum of opsonic amboceptors could be demonstrated :—

EXPERIMENT I.

Normal human serum was heated for 30 mins. at 60° C. (denoted "A"). A very thick emulsion of tubercle bacilli in 1 : 1000 salt solution was added in equal volumes to "A" and kept in contact therewith for 1 hr. 30 mins. at 37° C. The mixture was then centrifugalised (7000 revolutions per minute) for 1 hr., and the supernatant fluid pipetted off (denoted "B").

Equal volumes of "B" and fresh normal human serum were now mixed and kept in contact at 37° C. for 1 hr. (final fluid denoted "T").

The steps employed in the preparation of the control fluid were essentially similar to the above. Thus, heated normal serum was added in equal volume to salt solution (instead of to tubercle emulsion) and the mixture kept in contact for 1 hr. 30 mins. at 37° C. Equal volumes of this mixture and fresh normal serum were now taken, and retained in contact for 1 hr. at 37° (control fluid so obtained denoted "C").

The opsonic contents of "T" and "C" were now compared in the usual way, fresh tubercle and staphylococcal emulsions being employed. The result was as follows :—

	Bacilli per leucocyte.
Test fluid "T" + leucocytes + *B. tuberculosis*	0·5
Control fluid "C" + leucocytes + *B. tuberculosis*.........	2·3
	Cocci per leucocyte.
Test fluid "T" + leucocytes + staphylococci	1·4
Control fluid "C" + leucocytes + staphylococci	3·57

The difference between "T" and "C" was sufficiently striking, but it seemed desirable to re-test the two fluids with stronger emulsions of *B. tuberculosis* and staphylococcus. Accordingly, after standing in the cold room over-night at a temperature of 2° C., they were again compared, with the following result:—

	Bacilli per leucocyte.
Test fluid "T" + leucocytes + *B. tuberculosis*	0·8
Control fluid "C" + leucocytes + *B. tuberculosis*	4·5

	Cocci per leucocyte.
Test fluid "T" + leucocytes + staphylococcus	3·9
Control fluid "C" + leucocytes + staphylococcus	24·5

Thus the ratio of "C" to "T" in the case of tubercle rose after 16 hrs. from 4·6 : 1 to 5·6 : 1, and in the case of staphylococcus from 2·5 : 1 to 6·2 : 1.

Experiment II.

Fluids "T" and "C" were prepared in the way described above and their opsonic contents compared. Result:—

	Bacilli per leucocyte.
Test fluid "T"	2·6
Control fluid "C"	6·0

	Cocci per leucocyte.
Test fluid "T"	4·2
Control fluid "C"	17·2

After standing over-night in the cold room, they were re-tested with fresh emulsions of bacilli and cocci. Result:—

	Bacilli per leucocyte.
Test fluid "T"	1·0
Control fluid "C"	7·7

	Cocci per leucocyte.
Test fluid "T"	2·3
Control fluid "C"	9·0

The ratio of "C" to "T" thus rose after 16 hrs. from 2·3 : 1 to 7·7 : 1 in the case of *B. tuberculosis*, while in the case of staphylococcus it remained practically the same.

Experiment III.

In this experiment the heated serum and the salt solution were emulsified directly with *B. tuberculosis* in an agate mortar. Result:—

	Bacilli per leucocyte.
Test fluid "T"	1·2
Control fluid "C"	5·0

	Cocci per leucocyte.
Test fluid "T"	5·4
Control fluid "C"	15·9

Experiment IV.

Instead of *B. tuberculosis*, staphylococci were brought in contact with heated normal serum. The further steps in the preparation of "T" and "C" remained the same. Result:—

	Cocci per leucocyte.
Test fluid "T"	10·7
Control fluid "C"...............	15·7

(Opsonic content with regard to *B. tuberculosis* omitted.)

The difference between "C" and "T" is thus relatively small. A similar experiment was made, in which staphylococci, which had been heated at 60° C. for 1 hr. 30 mins., were passed through normal heated serum. Result :—

	Cocci per leucocyte.
Test fluid "T"...................	20
Control fluid "C"...............	18

So far, then, as these latter experiments are concerned, it does not appear that much, if any, inhibitory action on fresh normal serum is exerted by heated serum through which staphylococci have been passed. In view of the consistent results obtained with tubercle bacilli in the first series of experiments, it was important to determine whether saline solutions, through which bacilli have been passed, exhibited a similar inhibitory action on fresh normal serum.

A saline solution (1 : 1000) was digested at 37° with tubercle bacilli for 1 hr. 30 mins. and then centrifugalised. The extract so obtained was added in equal volume to fresh normal serum and kept at 37° C. for 1 hr. A control fluid was made by digesting the normal serum with salt solution (1 : 1000). The contents in tubercular opsonin of these two fluids were then compared, with the following result :—

	Bacilli per leucocyte.
Test fluid	5·4
Control fluid	7·7

Three similar experiments yielded the following figures :—

	Bacilli per leucocyte.		Cocci per leucocyte.
Test fluid	6·7	5	11·9
Control fluid	10·6	6	11·9

Extracts of tubercle bacilli in salt solution have, therefore, little, if any, inhibitory action on the tubercular and staphylococcal opsonins of fresh normal serum.

Conclusions.

Heated normal serum, through which tubercle bacilli have been passed, exerts a marked inhibitory action on the tubercular and staphylococcal opsonins of fresh normal serum. Such an action points to the presence in normal serum of opsonic amboceptors, the combination of which with the free receptors thrown off from the tubercle bacilli deviates the complement from the fresh normal serum, and thus inhibits phagocytosis. Further experiments of this nature are in progress.

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ON THE EXCRETION OF NITROGEN, CREATININE AND URIC ACID IN FEVER. By J. B. LEATHES.

It was observed by Folin that the absolute amount of creatinine excreted by any individual was the same, provided that his diet contained no creatine, whether that diet contained no more than 1 gramme of nitrogen or the usual full allowance. He argues from this that the excretion of creatinine, more strictly than that of nitrogen in all forms, or particularly of urea, should be taken as a measure of the true metabolism of the tissues[1].

Very similar views as to the significance of the excretion of urea, and therefore of the ordinary nitrogen estimations in urine, as an index of tissue metabolism, had it seemed to me been forced upon us by the work of Jacoby and of Lang[2]. This work showed that the amino acids formed in the digestion of proteid food are exposed, in the cells of the intestine and the liver and of other organs as well, to the action of an enzyme that removes the amino groups, presumably by a hydrolytic change, and so converts these compounds into non-nitrogenous substances. The ammonia thus set free may be supposed to be converted into urea in the liver and excreted in the urine at a time when the chemical energy of the organic compounds from which it was derived is still intact. The appearance therefore in the urine of nitrogen derived from proteid food tells us nothing certain as to what at the time has become of the essential organic compounds of which that proteid was composed, and nothing as to the proportion of the total energy set free in the body which has been derived from proteid sources. And to measure the true nitrogenous metabolism of the body tissues by estimating the nitrogen in the urine is clearly unjustifiable when proteid food is being absorbed.

Folin's conclusion referred to above was the result of comparing the proportions in which the different nitrogenous substances occur in the urine, when a diet of starch and cream containing only 1 g. of nitrogen was taken, with those observed when the usual amount of nitrogenous food was taken by the same individual. The nitrogen amounting to about 4 g. excreted daily on the starch and cream diet clearly must be for the most part derived from tissue metabolism. And the fact that of this nitrogen as much as 17 °/₀ is in the form of creatinine is very important and striking, and well able to carry the weight of argument that Folin proceeds to build upon it. But while the percentage of the total nitrogen which is excreted in the form of creatinine is in such circumstances so much increased, that which is excreted in the form of urea though considerably reduced is still found to be 60 °/₀, and therefore even so the principal form in which the products of tissue metabolism give up their nitrogen is still urea.

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[1] O. Folin. *Amer. Journ. of Physiol.* XIII. pp. 66 and 117. 1905.

[2] These views are discussed more fully in *Problems in Animal Metabolism*, p. 155 seq., John Murray, 1906.

In the interpretation of the nitrogenous metabolism of the tissues there are thus two facts of importance to be reckoned with: firstly that a part of the urea, perhaps the greater part of that excreted under ordinary conditions, has no more to do with tissue metabolism than the additional uric acid excreted after ingestion of uric acid itself or of other purine bases; it is simply formed from ammonia that has been rejected from redundant nitrogenous food-stuffs: and secondly that the creatinine is unlike the urea in that all of it is derived from the internal metabolic processes of the tissues, except such small fractions of it as are due to the creatine gratuitously introduced into the system with the food. These two facts show that in the interpretation of tissue metabolism we shall have to assign more importance to creatinine and less to urea than the relative proportions in which they occur in ordinary urine would indicate.

But it does not follow of course that increased tissue metabolism will show itself principally in increased creatinine excretion, nor even necessarily in such an increase at all. Urea, uric acid and ammonia as well as creatinine are all found in the urine when the nitrogen in the urine is almost entirely derived from the tissues and not the food, and an increased tissue metabolism may show itself in an increased output of some of these more than of others, or even unaccompanied by any increase in the output of others. It may be taken as probable that the metabolic processes leading to the production of these different
ogenous excreta are themselves different, and till we know what the
cance in metabolism of each of the excretory substances is we
foretell what effect any departure from the normal conditions
ve on the relative proportions in which they occur in the urine.

In fever, it has long been known, the excretion of nitrogen is high and independent of the amount admitted into the body with food. This nitrogen must therefore be traced to the breakdown of nitrogenous substances which have formed part of the body. And the investigation therefore of the nitrogen excretion in fever in the light of the facts referred to above acquires special interest.

It is easy to determine what proportion of the total nitrogen excreted occurs in the form of creatinine in the urine of patients suffering from febrile diseases, who are not given any meat, and to compare this with the figures from the normal urine of healthy subjects.

In normal individuals on a diet containing full amounts of nitrogen but no meat Folin found values for this ratio ranging from 3·5 to 4·5 %[1]. Van Hoogenhuyze and Verploegh, who used the same method for estimating creatinine as Folin, give tables from which it appears that in their experiments the ratio of nitrogen excreted as creatinine to the total nitrogen, $\left(\frac{\text{CN}}{\text{TN}}\right)$, did not sink below 4 % and ranged from that to 6 % even with diets containing 100 g. of proteid or more[2]. In two normal individuals taking no meat, but a diet that was otherwise varied and gave a nitrogen output amounting to about 14 g. daily, I found in one case on 4 successive days the values 5·21, 5·03, 4·89 and 5·75 %, in the other 5·73, 5·84 and 5·26 %.

This ratio $\frac{\text{CN}}{\text{TN}}$ was then determined in the following cases of febrile diseases among patients in St Thomas's Hospital.

I. A. O. Adult: Rheumatic Fever.

	Maximum temp.	Total N.	Total creatinine	$\frac{\text{CN}}{\text{TN}}$ %
4th day of fever.	101·6	21·37	1·35	2·37
5th ,,	101·8	21·79	1·54	2·63
6th ,,	100·6	18·89	1·32	2·61
7th ,,	101·8	19·86	1·34	2·51
10th ,,	99·4	11·73	1·29	4·79

II. T. W. Age 5: Pneumonia.

	Maximum temp.	Total N.	Total creatinine	$\frac{\text{CN}}{\text{TN}}$ %
6th day of fever.	104·4	—	—	2·28
7th ,,	103	6·90	0·39	2·09
8th ,,	102·4	6·11	0·24	1·43
9th ,,	102·6	—	—	1·82
10th ,,	99·6	—	—	2·34

Some of the urine was lost on three out of the five days, so that it was possible to give the total output for the 24 hours only on two of the days.

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[1] Folin, *loc. cit. supra.*

[2] Van Hoogenhuyze and Verploegh. *Zeits. f. physiol. Chem.* XLVI. p. 415. 1905.

III. J. B. Adult: Ulcerative Endocarditis.

Maximum temp.	Total N.	Total creatinine	$\frac{\text{CN}}{\text{TN}}$ %
101·6	12·9	1·215	3·49
102·4	11·37	0·942	3·08
103·2	11·14	0·966	3·30

These figures show that though probably in each case and certainly in the first two there was increased tissue metabolism, the proportion of the nitrogen leaving the body in the form of creatinine was low, in all probability lower than usual in health.

But it is difficult to decide this on patients who are already ill before they come under observation, and whose diet when they are not ill it is difficult to control. Accordingly I started to take myself a diet which contained no meat nor soups made from meat but was otherwise not restricted. The morning and mid-day meals were composed of bread, butter and eggs, the evening meal of milk soup, vegetables, omelette, macaroni and cheese. Approximately the same quantity of each article of food was taken each day. Tea was taken three times a day and coffee once; no other fluids were drunk. The urine was taken and measured at intervals during the day and each specimen analysed by itself. The total excretion for each 24 hours, beginning with the last day on which meat was included in the diet, was as follows:

	Total N.	Total creatinine	$\frac{\text{CN}}{\text{TN}}$ %
July 5 (diet with meat)	15·80	2·22	5·4
,, 6 (diet with no meat)	15·31	2·07	5·03
,, 7 ,, ,,	14·34	1·94	4·89
,, 8 ,, ,,	13·07	1·95	5·75

On the 9th at noon 1·75 c.c. of the anti-typhoid vaccine prepared at the laboratories of the Army Medical Staff were injected into the flank. The usual constitutional symptoms of the reaction to the vaccine came on in the evening, loss of appetite, headache, pains in the legs, and buzzing in the ears. The rectal temperature rose gradually from 99·8 at 2 p.m. to 102·7 at 11.30. At 1 a.m. it was 102·2 and at 2 a.m. 102·0; and then after about 4 hours' sleep was 101·0 at 7 a.m., and continued to be slightly raised till the afternoon when at 4.30 it was 100·5 which for that time of day during laboratory work is barely above a normal figure. The figures for July 9 and 10 to complete the above series were as follows:

	Total N.	Total creatinine	$\frac{\text{CN}}{\text{TN}}$ %
July 9	20·94	2·35	4·17
,, 10	14·12	1·87	4·70

There was thus on the day on which the temperature went up an increase of about 20°/₀ in the output of creatinine, but an increase in the total nitrogen output of more than 50°/₀, and consequently a diminution in the percentage of the total nitrogen formed by the creatinine nitrogen. How closely in point of time the rise in the excretion of nitrogen and of creatinine coincides with the rise of temperature is shown by the curves in Fig. 1. These curves were

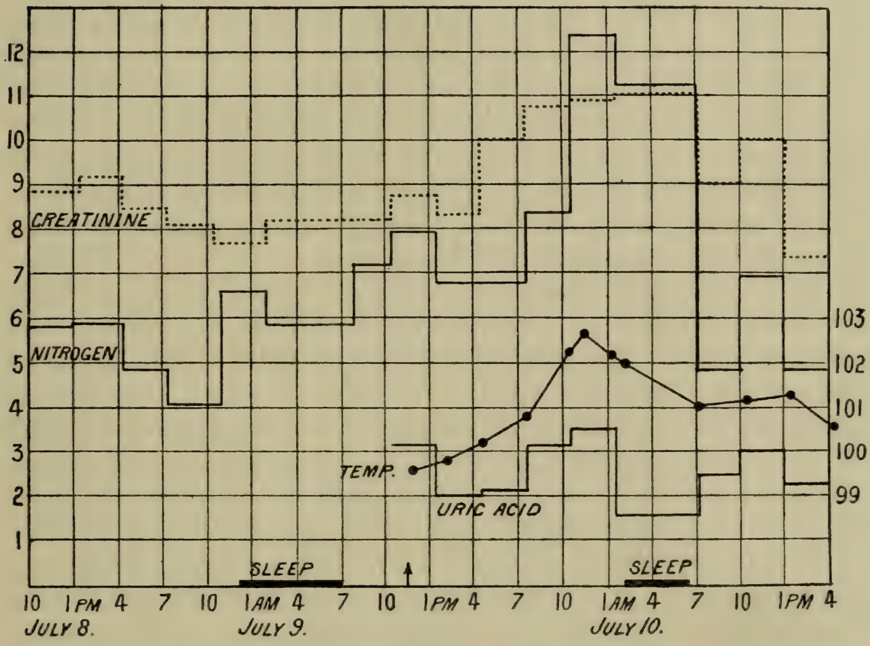

Fig. 1. Showing the rate of excretion of nitrogen, in decigrammes per hour, and of creatinine and uric acid, in centigrammes per hour, for the day preceding and the day following the injection of anti-typhoid vaccine. The time of the injection is marked by the sign ↑ and the course of the rectal temperature is indicated from that time onward.

obtained by calculating the hourly rate of excretion of nitrogen, creatinine and uric acid for each of the periods of the day preceding and following the injection which caused the febrile attack (Fig. 1). The variations in the rate of excretion of nitrogen and creatinine on the first day shown in the figure are typical for the conditions of this experiment: that is to say, the nitrogen excretion is highest during the first hours of sleep, declines during the latter part of the night, rises and is high through the first half of the waking hours and declines again in the evening. After the injection however the evening decline is replaced by a sharp rise corresponding exactly with the rise of temperature. The creatinine excretion is normally lowest at night and is not affected by the rapid excretion of nitrogen that occurs in the first hours of sleep. It rises during the day and reaches its maximum in the early afternoon[1]. But after the injection the rate at which creatinine was excreted rose, instead of falling, at night and reached its maximum at about the time when the temperature was highest. Similarly with the uric acid, the excretion rose and was very high between the hours of 7 p.m. and 1 a.m. when it is normal for the excretion of this substance to be less active than at any other time of the day. The uric acid in this experiment, however, was not determined except for the times indicated in the figure, and these determinations were made under difficulties as an afterthought. The effect of fever on the excretion of uric acid comes out more clearly in a later experiment which was carried out expressly for showing this.

In the later experiments special care was taken to obtain the curve of excretion under normal conditions with which to compare that obtained in fever. The irregularities traceable to the diuretic action of tea and coffee were eliminated, and also those due to the lack of uniformity in the distribution through the day of the nitrogen intake. Food was taken six times a day at intervals of 3 hours and all the six meals were identically the same both as to fluid and solid components. The urine for each 3-hourly period was analysed separately. But even so, remarkable variations in the rate of excretion at different times of the day occur, and recur daily; these have been described and in part discussed in an earlier paper. The regularity with which these variations occur on a series of normal days makes it possible to construct a curve of the rates of excretion normal for a particular diet with which to compare the rates induced by abnormalities such as a febrile attack.

In the first experiment on this plan, after 5 normal days on a fixed diet composed of milk, bread and butter, a second dose of the anti-typhoid vaccine equal in amount to the first was injected about 5 weeks after it. But this was not followed by any certain rise of temperature not was there any marked alteration in the rate of excretion of either nitrogen, creatinine or uric acid. The immunity established by the first dose was proof against a second dose of the same strength 5 weeks later and no reaction was to be noted except the local one and in a slight degree some of the subjective symptoms, headache and pains in the legs. Accordingly 4 days later a dose of the plague vaccine prepared at the Lister Institute was injected, which had been proved in other subjects to give a marked reaction. But this was also followed by no definite symptoms, no fever and no change in the excretions. This result was remarkable, for in a sample of blood taken 13 days later the opsonic index for plague bacilli was found by Dr Boycott to be 1·6, showing that in spite of the absence of constitutional symptoms the vaccination had not been without effect.

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[1] Curves taken from the average of two series of days on different diets illustrating these diurnal variations in the rates of excretion were given in a former paper on 'Diurnal Variations in the Excretion of Uric Acid,' this *Journal*, vol. xxxv. p. 125.

The following protocol gives the figures for the periods of 24 hours each, beginning from the day on which the diet was first taken:

	Total N.	Total creatinine	Uric acid	Remarks
Aug. 12	14·65	2·05	—	
13	15·96	2·04	—	
14	17·90	1·99	—	
15	18·60	2·10	—	
16	19·64	2·16	0·55	
17	19·67	2·17	0·64	1·75 c.c. Typhoid Vaccine at the beginning of this period of 24 hours at 1 p.m.
18	20·37	2·05	0·635	
19	17·30	2·02	0·59	
20	17·77	2·06	0·57	
21	18·52	2·12	0·59	1·75 c.c. Plague Vaccine at the 5th hour of this period of 24 hours.
22	18·76	2·10	0·60	

After the typhoid vaccination the local reaction was marked as on the former occasion by redness and tenderness over an area about 4 or 5 ins. in diameter. The temperature taken at intervals of about 3 hours throughout the 24 hours did not rise above 100·2. This is not more than a degree above the normal for rectal temperatures at the hours at which it was observed, viz. 3 a.m. and 5 a.m. The details of the urine analyses show that in none of the three-hourly periods was there any more marked departure from the normal average rates for those periods than that indicated by the sum of the excretions for the whole 24 hours given in the column of figures above.

After the plague vaccination the rectal temperature was again 100·2 at 5 a.m., 11 hours after the injection. At 10 a.m. it was 100·9, but that is a temperature which may frequently be observed during the active hours of the day.

For the next experiment a different diet was taken consisting of plain biscuits, butter and water, taken as before in equal amounts every 3 hours. The daily intake of nitrogen was about 5 g., the daily output, after it had become approximately constant, was about 7·5 g., the heat equivalent of the food was also considerably less than in the earlier experiment, so that there was some loss of weight amounting in 13 days altogether to about 1 kg.

On the last day but one a larger dose, 4·6 c.c., of the same anti-typhoid vaccine as that taken in the earlier experiments was injected into the flank as before, the interval between this dose and the previous one being about 5½ weeks.

Protocol.

On Sept. 24, 6 p.m., 4·6 c.c. anti-typhoid vaccine injected.

10 p.m. The local reaction was pronounced with redness and tenderness.

11.15 p.m. A definite but not severe rigor lasting about 20 mins., shortly afterwards the temperature was 101·2.

Sept. 25, 1.30 a.m. Temp. 103·0, headache and pain down the legs.

4.30 a.m. Temp. 102·8, less headache, but sciatic nerves painful on stooping.

7 a.m. Temp. 102.

10 a.m. Acute symptoms passed off, leaving weakness and aching. The usual meals were taken at 7 and 10 a.m. and at 1 p.m., but the other three meals due that day were dropped, though the water was taken as usual; the high nitrogen excretion on the night of the 25—26th may be partly due to this. In the evening of the 25th the particularly high nitrogen excretion between the hours of 7 and 10 p.m. is also to be explained in part as the usual effect of an hour and a half's deep sleep.

In the period preceding the injection on 7 normal days, after the irregularities of the first few days on which the diet was taken had disappeared, the following figures were obtained for the excretion of nitrogen, creatinine and uric acid:

	Total N.	Total creatinine	Total uric acid
Maximum	8·04	2·22	0·50
Minimum	6·81	1·88	0·38
Average for 24 hrs.	7·63	2·07	0·44

In the 24 hours from 10 p.m. on the 24th to 10 p.m. on the 25th the corresponding figures were:

9·76	2·24	0·71

The details of the excretion in each of the periods of the day are given in Fig. 2 in which the dotted lines indicate the average rate of excretion in each period of 3 hours as obtained from the normal days on this diet, and the dotted temperature curve the temperatures observed during a normal night without fever. The unbroken lines show the rates observed in the corresponding periods after the injection. In order to avoid superposition of the curves, those for the total nitrogen are drawn throughout 2 divisions on the diagram higher than they actually should be. The values are given in centigrammes for creatinine and uric acid, and in decigrammes for nitrogen.

The most striking departure from the average rates is in the case of the uric acid: the rate between the hours of 10 p.m. and 4 a.m. being nearly 4 times the average rate for that period of the day and considerably higher than the maximum rate for any period of the day.

The rate of creatinine excretion was during these hours also higher than the normal maximum although normally these hours are those at which the rate is slowest. The actual increase in the rate of excretion of this substance during the night was about 25 °/₀ above the average for that period of the 24 hours.

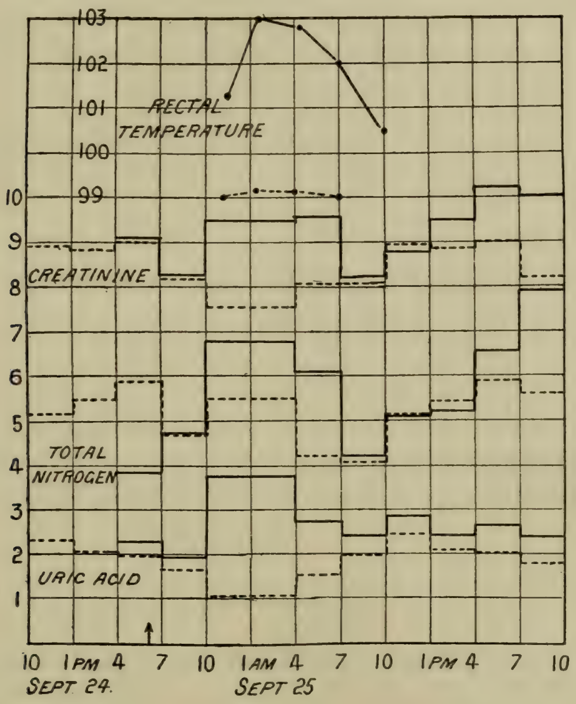

Fig. 2. Dotted lines show average rates of excretion taken from the observed rates of six normal days on this diet. Unbroken lines, the actual rates for the corresponding periods of the day after the injection of anti-typhoid vaccine, which took place at the time indicated by the sign ↑.

The total nitrogen excretion during the 9 hours of the night, when the temperature was raised two degrees or more above the normal, was also considerably above the average rate, the increase amounting to nearly 40 °/₀.

To return in conclusion to the point from which these experiments started, namely the proportion of the increased nitrogen output accompanying fever which appears in the form of creatinine, the first experiment showed that the ratio $\frac{\mathrm{CN}}{\mathrm{TN}}$ fell in the 24 hours in which the rise of temperature due to the typhoid vaccine occurred to 4·17 %, having averaged on the 4 previous days 5·22 %: the last experiment, in which of course, owing to the small amount of nitrogen in the food, this ratio had a higher value, gave a fall from the average of 9·8 % on the 9 previous days, to 8·4 % on the day in which the febrile attack occurred. These results confirm the evidence obtained from patients in hospital. The conditions of the last experiment however allow us to compare the ratio more exactly at the hours at which the temperature was raised. Thus:

	From 10 p.m. to 4 a.m.	From 4 a.m. to 7 a.m.	From 7 a.m. to 10 a.m.
Average value for $\frac{\mathrm{CN}}{\mathrm{TN}}$ %	7·8	13·0	14·2
Value during fever	7·4	8·7	13·2

If we take the similar ratio for uric acid the figures are

Average value for $\frac{\bar{\mathrm{U}}\mathrm{r.\,N.}}{\mathrm{T.\,N.}}$ %	1·0	2·1	3·3
Value during fever	2·6	2·3	3·5

While therefore the proportion of the nitrogen excreted during the fever which appeared as uric acid was very considerably increased, the proportion that appeared as creatinine was consistently diminished.

CONCLUSIONS.

In these experiments the increased nitrogenous metabolism accompanying fever was found to involve an increased output of both creatinine and uric acid, as well as of total nitrogen. But the increased output was most marked in the case of uric acid, and least marked in the case of creatinine.

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XII. THE PATHOLOGICAL HISTOLOGY OF THE SPLEEN AND LIVER IN SPONTANEOUS RAT-PLAGUE, WITH OBSERVATIONS ON THE EXPERIMENTAL INFECTION.

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(PLATES VIII and IX.)

The pathological histology of plague in man and experimental plague in animals has already been the subject of several researches, notably those of Aoyama (1896), Yamagiwa (1897), Albrecht and Gohn (1897), Dürck (1904) and Hamdi (1904) in the case of human plague, and of van der Stricht (1897), Honl (1897), Lustig and Zardo (1897), Babes and Livadite (1897), Albrecht and Gohn (1900) and Sata (1900) in the case of experimental plague.

The guinea-pig and rat have been mainly employed as experimental animals owing to their great susceptibility to infection, but in view of the natural occurrence of plague in the latter animal and the important part it plays in the propagation of human plague, it is highly essential that we should have all details regarding the histology of the natural rat infection even though the differences between such and the experimental form may be presumably insignificant.

The pathological histology of natural plague infection in rats has been referred to only by Albrecht and Gohn (1900) who gave brief histological details of the organs of five rats which had died of plague in Bombay. Ogata (1897) also contributed a few observations on the subject.

It is to fill up this lacuna in our knowledge of spontaneous rat plague that the following histological examinations have been made from material collected in Bombay by the Plague Commission. The primary cause, however, of this inquiry was the elucidation of the peculiar characteristic post-mortem appearances of the spleen and liver, to which reference has been made in another article in this number. For this purpose, therefore, only the spleens and livers from the various cases were forwarded.

It was felt, moreover, that a more minute investigation of the histological changes might throw some light on the conditions leading up to the chronic form of rat plague which is associated with the presence, in the spleen particularly, of encapsulated abscesses.

To add completeness to the work and for purposes of comparison the organs of several rats which had been inoculated in Bombay with virulent plague, were submitted to detailed examination by similar methods. Finally one or two cases of chronic experimental plague in vaccinated rats were investigated histologically. The results obtained in these experimental cases will be discussed in Part II of this paper.

PART I.

Spontaneous Rat Plague.

The very brief histological details recorded by Albrecht and Gohn may be here summarised:—

Rat I. *Spleen*:—Numerous haemorrhages were present in the pulp. Necroses had also commenced especially at the periphery of the nodes. Bacilli were scarce. *Liver*:—Showed cloudy swelling. Bacilli scarce.

Rat II. *Spleen*:—Pulp was haemorrhagic and infiltrated with polynuclear leucocytes. The nodes were surrounded by a fine or coarse network of connective tissue. Bacilli were numerous. *Liver*:—Liver cells showed karyorrhectic nuclei in many cases. The capillaries contained enormous masses of bacilli.

Rat III. *Spleen*:—Pulp contained large amounts of nuclear débris especially in the vicinity of the nodes. *Liver*:—Showed degeneration of parenchyma. Capillaries were full of bacilli.

Rat IV. *Spleen*:—Pulp presented commencing necroses. Bacilli numerous.

Rat V. *Spleen*:—Numerous haemorrhages and necroses in the pulp. The peripheral portions of the nodes and the adjoining pulp-tissue were transformed into a coarse network staining intensely with eosin.

Rat VI. *Spleen*:—Showed haemorrhages and nuclear disintegration. Bacilli very numerous. *Liver*:—Liver cells showed fatty degeneration. Bacilli very abundant in the capillaries.

Ogata (1897), during the epidemic of plague in Formosa, obtained six rats which had died of plague. Referring to the condition of the organs he merely remarks that the spleen was much swollen and the liver congested. Small haemorrhages were also present in the liver and bacilli were numerous, as also in the spleen and glands.

From the above summary, it will be evident that the grosser lesions only have been recorded, possibly owing to the difficulty of procuring material fresh enough for detailed histological examination. The organs at my disposal were the spleens and livers of thirteen cases of rat plague. These had been fixed in Orth's fluid and sent to this country in spirit. The tissues were embedded in paraffin and the stains chiefly employed were Unna-Pappenheim's methyl-green-pyronin and Ehrlich's haematoxylin and orange-rubin. The former stain proved eminently satisfactory for the demonstration of plague bacilli and plasma cells.

Rat I. *Protocol* :—Primary bubo—left axillary. Congestion of spleen and subcutaneous tissues. Bacilli present in bubo, heart blood and spleen.

Spleen :—Capsule : shows no pathological changes.—Nodes : The nodes are few in number but regular in outline. Karyorrhexis of the lymphoid cells of the node is a marked feature and a large amount of nuclear detritus is lying either free or included in large endothelioid phagocytic cells. Some of these particles show their cytoplasmic origin by taking up the pyronin stain. Large mononuclear cells of endothelioid type are abundant throughout the node, many of them presenting mitotic figures (see Plate VIII, Fig. 2). The protoplasm of these cells stains a deep red, the nucleus being rather vesicular. No bacilli were detected within the node though a specially rich zone of them was present in the perinodal lymph sinus.—Pulp : Extravasation of red cells was very marked and plague bacilli were fairly uniformly distributed and in large numbers. No necrotic foci were present. Rows of plasma cells, many showing mitosis, were arranged in the sheaths of the trabecular vessels. *Liver* :—The protoplasm of the liver cells was coarsely vacuolated. Necrotic foci, which are the outstanding feature of plague livers, were in this case few in number and of very small size, in some cases only two or three liver cells being involved. Within these necrotic areas were a few vesicular nuclei and occasionally a small group of bacilli lying in what remained of the intraacinar capillaries. Enormous numbers of bacilli were present in the liver capillaries. Many of the liver cell-nuclei showed karyorrhexis.

Rat II. *Protocol* :—Primary bubo—left submaxillary. Spleen, lungs, and subcutaneous tissues congested. The surface of the liver had a mottled appearance. Bacilli in spleen and bubo.

Spleen :—Subcapsular haemorrhage was considerable.—Nodes : These were greatly diminished in number. Large mononuclear endothelioid cells were numerous, the small lymphoid cells showing extreme karyorrhexis. In some nodes were small necrotic foci containing large endothelioid cells and nuclear detritus, similar to those described in the diphtheritic spleen (see Waschkewitsch *Virch. Archiv*, Bd. 159, 1900).—Pulp : There was great congestion of the pulp sinuses accompanied with red cell extravasation. Around the trabecular vessels were many plasma cells showing extreme karyorrhexis and pyknosis (see Plate VIII, Fig. 5). The cells of the spleen pulp presented as a whole only a slight degree of karyorrhexis and no actual necrotic foci were observed. Bacilli occurred in swarms throughout the pulp especially in the areas of blood extravasation. *Liver* :—The protoplasm of the

EXPLANATION OF PLATES VIII AND IX.

Plate VIII. Fig. 1. Portion of necrotic focus of liver (natural rat plague). *a*=Blood in capillaries. *b*=Remains of liver-acini. *c*=Liver cell showing vacuolar honeycomb degeneration. *d*=Mononuclear cell in capillary.

Fig. 2. Portion of malpighian body (natural rat plague) showing large mononuclear endothelioid cells. *a*=Mitosis of the same. A degenerated endothelial cell containing nuclear detritus is also seen.

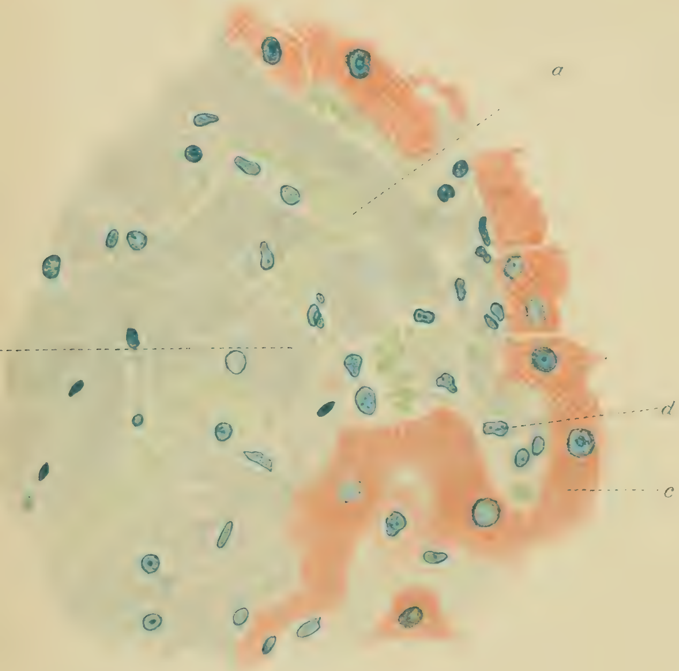
Fig. 3. Portion of spleen in chronic experimental rat plague. *a*=Giant-cell (of tubercular type). *g*=Commencing agglomeration of nuclei to form giant-cell. *b*=Megakaryocyte. *d*=Granulation spindle cell. *c*=Rows of plasma cells. *f*=Leucocytic débris round bacilli. *e*=Degenerated bacilli.

Fig. 4. Portion of liver (natural rat plague). *a*=Megakaryocyte in capillary. *b*=Liver-acinus.

Fig. 5. Portion of spleen-pulp (natural rat plague) showing bacilli and pyknotic plasma cells of all types, and effused red cells.

Fig. 6. Giant-cell (of Langhans' type) from periphery of malpighian body (experimental rat-plague). The drawings are made from five successive sections through the same cell. Note the grape-like nuclear agglomeration in the 4th drawing.

Plate IX. Photomicrograph of liver necroses (natural rat-plague).



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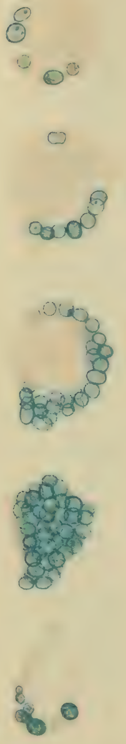

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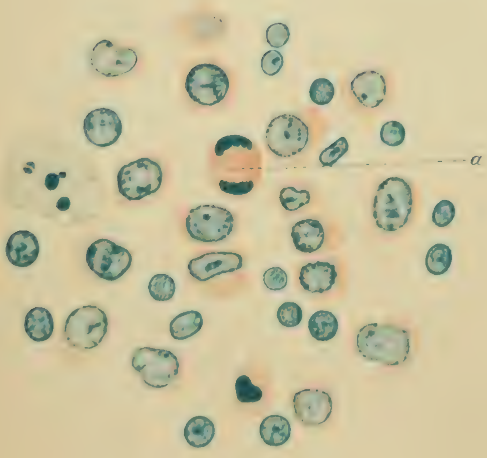

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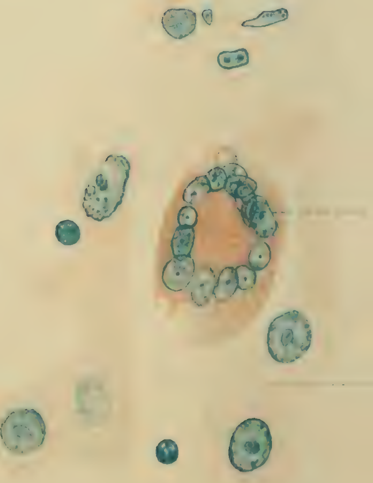

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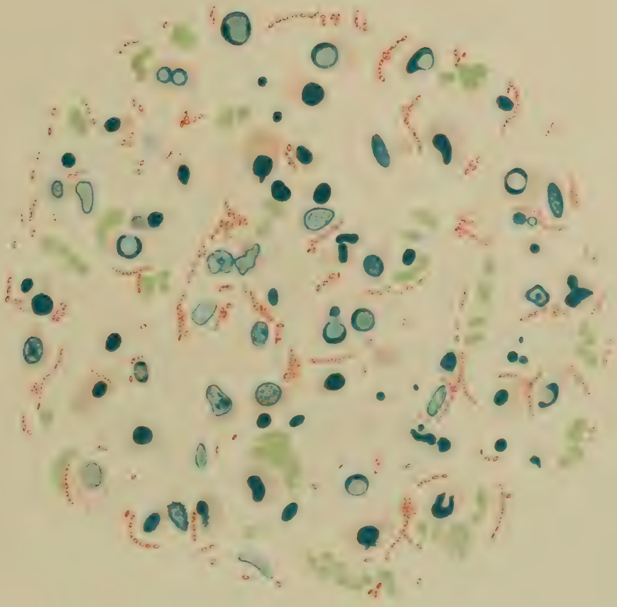

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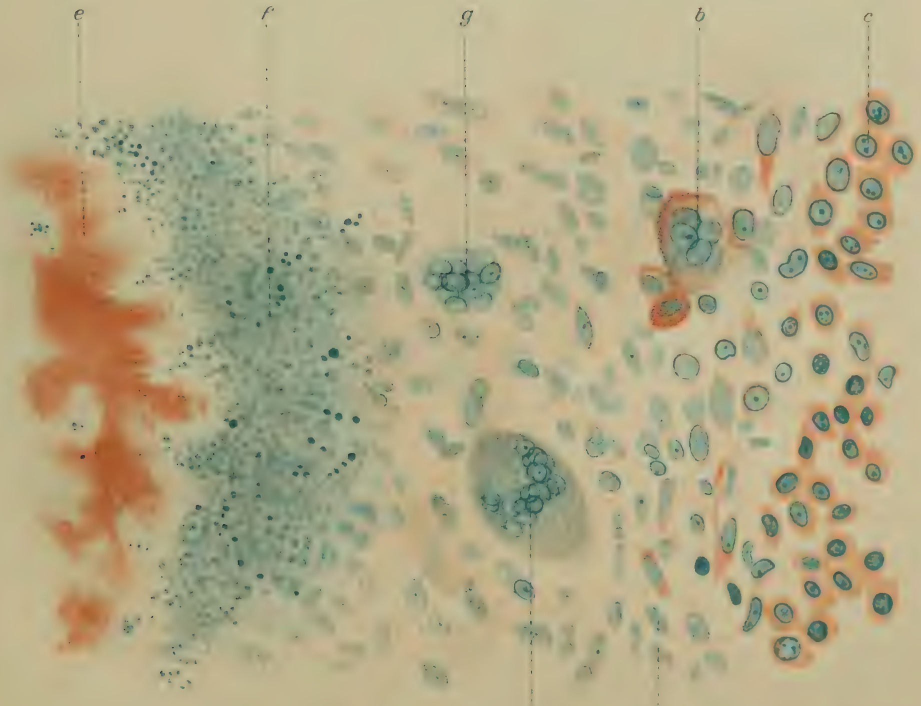

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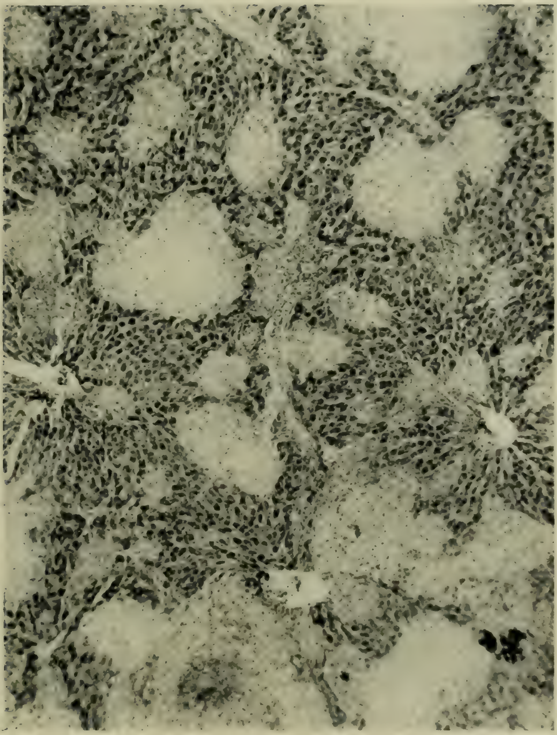

liver cells was finely vacuolated but not fatty. Acinar necroses were few in number and of very irregular contour. In the capillaries of the necrotic area, bacilli were still present. The portal vessels and intraacinar capillaries were extremely congested and contained large numbers of bacilli.

Rat III. *Protocol* :—Primary bubo—right submaxillary. The liver had a granular appearance and presented minute haemorrhages on the serosa. Bacilli present in heart blood, spleen and bubo.

Spleen :—The capsule presented no marked changes.—Nodes : These were few and of irregular shape and size and showed the characteristic endothelioid proliferation with numerous mitoses. At the periphery of the node plasma cells were abundant. A marked feature was the presence of a reticular zone encircling each node. The few pulp cells remaining in this zone showed extreme karyolysis and bacilli were specially abundant.—Pulp : The pulp showed great congestion with haemorrhages and polynuclear infiltration. No definite necrotic foci were seen although bacilli occurred in swarms. Plasma cells were numerous in the sheaths of the vessels and there was a very pronounced catarrh of the vascular endothelial cells. *Liver* :—Focal liver cell necroses were fairly numerous especially in the subcapsular area. They were apparently of recent origin and contained many extravasated red blood corpuscles with bacilli still lying in the capillaries. The liver cells showed fine vacuolation of their cytoplasm. Bacilli were not quite so numerous in the vascular system of the liver as in the previous cases.

Rat IV. *Protocol* :—Primary bubo—right submaxillary. The serosa of spleen and liver had a granular appearance. Bacilli few in heart blood, spleen and bubo.

Spleen :—The capsule was markedly thickened and oedematous, the subcapsular lymph space showing extreme endothelial catarrh.—Nodes : The nodes were greatly diminished in number and in some cases contained rounded areas with vacuolated endothelial cells and nuclear débris. Many lymphoid cells had undergone karyorrhexis.—Pulp : Even with low magnification large clumps of bacilli could be seen throughout the pulp, each surrounded by a zone of karyorrhectic nuclei. The condition was thus one of multiple small abscess formation. Around the nodes and vessel-sheaths plasma cells and vesicular epithelioid cells abounded. The karyorrhectic focus was immediately surrounded by a zone of vesicular epithelioid nuclei and this again by a barrier of plasma cells. These epithelioid cells showed a slight tendency to nuclear grouping but no fully formed giant-cells of tubercular type were met with. *Liver* :—Liver cell necroses were present in enormous numbers and in fact little healthy liver tissue was left (see Plate VIII, Fig. 1 and Plate IX). Some foci contained only red blood corpuscles, with no traces of bacilli remaining, while others contained a central clump of bacilli surrounded by karyorrhectic nuclei or proliferating epithelioid cells. The production of some necroses by vascular bacillary emboli was quite patent though in others such a connexion could not be traced. Among the vesicular nuclei surrounding one focus a fully formed giant-cell of tubercular type was detected. In the healthy liver capillaries, bacilli were scarce and evidently degenerated, their remains being taken up by the endothelial cells of the vessel wall. A very interesting feature was the presence in the dilated capillaries of great numbers of epithelioid cells some of which were undergoing mitosis. An occasional giant-cell of the splenic megakaryocyte type was detected in the capillary lumen. Round the portal vessels in Glisson's capsule small lymphomata were sometimes met with.

Rat V. *Protocol* :—Congestion of subcutaneous tissue, lungs and spleen.—Liver fatty and granular. No bacilli in heart blood, spleen or liver.

Spleen :—The capsule is very oedematous.—Nodes : There is no diminution in the number of malpighian bodies, each containing many endothelioid cells with plasma cells at the periphery. Among the latter, very many mitotic forms were observed. A perinodal zone of reticular tissue with few nuclei was a constant feature, forming a species of capsule for the node.—Pulp : The pulp was extremely congested but only a few bacilli were detected in small groups lying among the extravasated red blood corpuscles. Many necrotic foci were present. These at times consisted of karyorrhectic nuclei and detritus or of vesicular epithelioid cells. The plasma cell reaction was very conspicuous. Pigment cells containing haemosiderin and catarrhal endothelial cells occurred in fairly large numbers. *Liver* :—Very numerous sharply demarcated necroses were present in all stages of development. In some of them, red blood corpuscles were the predominant elements, while in others the endothelial cells of the capillaries showed a marked proliferation. A few bacilli could still be detected in these foci. Elsewhere, the capillaries of the liver contained here and there small bunches of bacilli which appeared to have undergone degenerative changes. The phagocytic endothelial cells frequently contained such bacillary débris.

Rat VI. *Protocol* :—Double pelvic bubo containing swarms of bacilli. No bacilli in heart blood or spleen.—Liver coarsely granular and spleen congested.

Spleen :—The capsule was oedematous and the subcapsular area very much congested, the endothelial cells actively proliferating.—Nodes : These were much diminished in number but presented only slight pathological changes.—Pulp : The pulp was greatly congested. There were no definite areas showing karyorrhexis of the pulp cells but here and there could be seen foci in which the nuclei had disappeared or had become vesicular. Pyknotic plasma cells were present in moderate numbers. No bacilli could be demonstrated. *Liver* :—The liver cells looked fairly healthy. Focal necroses were moderate in number, a few remains of bacilli being still apparent in them. The portal vessels were extremely congested. Free bacilli were never seen in the capillaries but the endothelial cells contained here and there what appeared to be bacillary remains.

Rat VII. *Protocol* :—Primary bubo unknown. Liver fatty and granular. —Spleen granular. Many bacilli in spleen.

Spleen :—The capsule was oedematous and infiltrated with red cells, the superficial endothelium being catarrhal.—Nodes : These showed no important changes. No bacilli were detected in them.—Pulp : Large irregular necrotic areas were present showing no very definite boundary and frequently lying alongside a malpighian body. At the margins of these areas or inside them were wisps of bacilli. In one case, a vein leading to such a focus was completely blocked by plague bacilli for a considerable distance. Extraordinary numbers of bacilli were present in the pulp among the extravasated red cells and polynuclear infiltrates. Karyorrhectic and pyknotic plasma cells and catarrhal endothelial cells were specially numerous. *Liver* :—The liver cells throughout the lobule showed large protoplasmic vacuoles due to fat. Focal liver cell necroses were very numerous and sharply demarcated from the healthy liver substance. Some foci contained very few nuclei, while others contained numerous vesicular nuclei tending at times to coalesce. The contents of the capillaries were polynuclear cells, red cells and occasional degenerated bacilli. A few small lymphoid nodes were observed around the portal vessels in the large trabeculae. Large mononuclear endothelioid cells showing mitoses could be seen in these small lymphomata as in the splenic nodes.

Rat VIII. *Protocol* :—Primary bubo—left axillary. Liver fatty and granular. —Spleen faintly granular.—No bacilli in heart blood or spleen.

Spleen :—The chief feature was the presence of numerous pulp necroses in which only a few vesicular nuclei remained. These areas were invariably perinodal. Outside these areas the pulp cells showed slight karyorrhexis. Pyknotic plasma cells occurred in enormous numbers. Bacilli were exceedingly scarce and confined to the necrotic foci. *Liver* :—Cell necroses were very numerous and contained nuclear débris and vesicular epithelioid cells. Sometimes a few degenerated bacilli could be seen in them. Elsewhere, the liver capillaries contained numerous degenerated endothelial cells and karyorrhectic nuclei. Bacilli were very scarce and only in phagocytic endothelial cells. There was marked congestion throughout.

Rat IX. *Protocol* :—Primary bubo—left submaxillary. Liver fatty and granular.—Spleen granular.—No bacilli in heart blood, spleen or bubo.

Spleen :—In this case the areas of pulp degeneration were very numerous and extensive. The malpighian bodies were encroached upon and very little normal splenic tissue remained. In the degenerated areas were enormous masses of nuclear detritus with occasional degenerated bacillary clumps in the centre. Bounding these areas were rows of plasma cells in active division and catarrhal endothelial cells. Signs were already present of the replacement of these foci by spindle-shaped granulation cells formed from the actively dividing plasma cells. *Liver* :—Necroses were few but of all sizes. Some bacilli still remained in the capillaries of the necrotic area along with red cells. Throughout the liver substance, the capillaries were greatly dilated with red cells, desquamated endothelial cells containing nuclear detritus and large mononuclear cells suggesting a splenic origin. Typical giant cells of megakaryocyte type were also met with not infrequently lying in the capillary lumen (see Plate VIII, Fig. 4). It seems most probable that these cells, along with many of the types filling up the liver capillaries, have found their way to this organ from the spleen. Babes and Livadite (1897) noted the presence of similar giant cells in the guinea-pig's liver (in experimental plague), but apparently assigned to them a different origin. They write "Sehr bemerkenswerth ist das Auftreten von Riesenzellen mit gelappten Kern wohl auf Kosten gewisser Endothelien." Only a very few bacilli were present in the capillaries.

Rat X. *Protocol* :—No primary bubo. Liver fatty and granular.—Spleen congested.—No bacilli in heart blood, spleen or liver.

Spleen :—Not available for histological examination. *Liver* :—Liver cell necroses were few and of small size. The subcapsular region was mainly the site of these foci and there also bacilli were most frequent. The capillaries of the necrotic area were blocked by bacilli in many cases. Throughout the liver substance the capillaries were dilated with red cells and splenic elements as in the previous case. Megakaryocytes were also occasionally detected. Bacilli as a rule were scarce and many were included in the endothelial phagocytes.

Rat XI. *Protocol* :—Primary bubo—left axillary. Liver and spleen congested. Many bacilli in spleen and bubo.

Spleen :—The lymphoid tissue was greatly diminished. Large mononuclear endothelioid cells in active division were present in the nodes. Necrotic areas containing vesicular nuclei and bacillary débris were distributed throughout the pulp. Megakaryocytes, pyknotic plasma cells and catarrhal endothelial cells were specially abundant. An interesting feature was the presence of a large number of eosinophile cells. *Liver* :—Necroses were few but of all sizes. The capillaries were dilated as in the previous cases with splenic elements. Plasma cells were also numerous. A slight degree of perivascular lymphoid infiltration was noted in the portal spaces.

Rat XII. *Protocol* :—Liver coarsely granular and fatty, spleen finely granular.—Subcutaneous and pulmonary haemorrhages.—No bacilli in heart blood, few clumps in spleen.

Spleen :—The nodes were few in number and badly differentiated from the surrounding pulp.—Pulp : Small irregular necrotic foci were distributed throughout the pulp. The centre of each focus was occupied by swarms of bacilli and the periphery by vesicular nuclei, detritus and blood-pigment-carrying cells. The subcapsular area was infiltrated with red cells and polynuclear leucocytes. Megakaryocytes were exceedingly numerous especially in the neighbourhood of the necrotic foci. *Liver* :—Fatty infiltration of the liver cell-protoplasm was far advanced. Many nuclei also exhibited karyorrhexis. Only a few small necrotic foci were noted. The intraacinar capillaries were greatly dilated, their lumina being filled with small and large mononuclear cells and endothelial phagocytes.

Rat XIII. *Protocol* :—Lungs both consolidated. Gray hepatisation of upper and middle lobes of right lung.—Liver has a mottled appearance.—Spleen congested. —Swarms of bacilli in heart blood, spleen and bubo.

Spleen :—Malpighian bodies were numerous and of irregular form and size. Many karyorrhectic nuclei were present in the nodes along with large endothelial cells containing nuclear detritus.— Pulp : A few clumps of degenerated bacilli were seen here and there but no necrotic foci were in evidence. The cell types met with in the pulp were very varied, small and large mononuclear cells, endothelial cells, megakaryocytes and nucleated red cells. An interesting feature was the great abundance of coarsely granular eosinophile cells many of which showed mitotic figures. They occurred in greatest numbers in the subcapsular area. A few clumps of mast cells were also noted. Plasma cells were very scarce. In this case the pneumonia was evidently the main plague-lesion, while the spleen presented none of the characteristic changes observed in the foregoing cases. The splenic picture, in fact, was that associated with an actively functioning haemopoietic organ. *Liver* :—Unsuitable for histological examination.

Survey of the above cases.

The cases may be divided into two main groups :—

1. Those in which bacteriaemia of the spleen and liver is at a maximum and has been of recent development.

2. Those in which bacteriaemia is less prominent or is rapidly disappearing as a result of reactive tissue changes.

In the first group, the incursion of bacillary swarms into the spleen and liver has been accompanied by extensive haemorrhages and congestion of the pulp sinuses and liver capillaries. Definite abscess formation in the spleen has not had time to develop and focal liver cell-necroses are few in number, the latter being very largely confined to the subcapsular region.

In the second group, definite abscess formation in the spleen is far more frequent and is accompanied by extensive reactive changes on the part of the plasma cells. The reduction in the amount of lymphoid tissue is due in great measure to the perinodal distribution of degenerated pulp areas. A barricade of plasma cells separates the lymphoid tissue of the node from the necrotic zone. Proliferation of the large mononuclear endothelioid cells of the node is a noteworthy feature. Dürck (1904) has described a similar change in the splenic nodes in human plague.

In the liver, focal necroses may be so numerous that little healthy liver tissue remains. The demonstration of bacilli in the central capillaries of these foci was made in nearly every case and frequently actual bacillary embolism was noted. The formation of giant-cells of Langhans' type in the neighbourhood of necrotic foci is also of great importance, although in some of the cases these cells had not reached their full development. It can readily be conceived how, providing the animal lives long enough, the reaction of the fixed tissue cells may proceed to complete encapsulation of abscess areas and so bring about a more or less chronic condition. So far I have had no opportunity of examining histologically such cases of chronic spontaneous rat plague, but it is hoped that suitable material of this kind may soon be available. The chronic experimental case, which will be described later, gives however a very clear notion of the later stages in this process of abscess-encapsulation.

Frequent mention has been made in the protocols of a granular and mottled appearance of the liver. The spleen has also been described as granular in some cases. In the case of the liver such changes are readily accounted for by the distribution of the haemorrhages and the focal necroses together with the fatty changes in the liver cells. It must be understood, however, that a peculiar, honeycomb-like vacuolar degeneration of the liver cell protoplasm was far more frequent than an actual coarse fatty infiltration. The granular appearance of the spleen is due partly to endothelial catarrh and partly to subcapsular changes.

With regard to the disappearance of bacilli from the intraacinar capillaries of the liver it appears that phagocytosis by endothelial cells is largely responsible. Indeed in some cases no free bacilli were demonstrable. The presence of large giant-cells of megakaryocyte type in the liver capillaries of some cases is highly interesting: in such cases the capillaries generally contained so many extraneous cell elements that one is forced to assign to them a splenic origin.

Finally, with regard to the distribution of plague bacilli in the spleen, it was exceedingly rare to find the organisms in the interior of the nodes. Yet, though bacilli were absent, karyorrhexis of the nodal cells was frequently far advanced. Toxaemia must then be largely responsible for the alterations in the nodes, as we know that analogous changes take place in the malpighian bodies after the inoculation, for instance, of diphtheria toxin.

PART II.

Experimental Rat Plague.

The following is a brief *résumé* of our knowledge regarding the histology of the spleen and liver in experimental plague.

Spleen. In the guinea-pig van der Stricht (1897) noted a diminution in the size of the malpighian bodies with dilatation of the capillaries of the node, leading to actual rupture. Abscesses occurred in the pulp but did not affect the nodes. The splenic megakaryocytes were very numerous and the capsule of the organ was infiltrated with white corpuscles. Honl (1897), working with the same animal, found an enlargement of the follicles and extreme congestion of the pulp. Bacilli occurred in "zoogloeal" groups round which were numerous fragmented cell elements. Lustig and Zardo (1897) working with rats, mice, guinea-pigs and rabbits noted that the periphery of the follicle was the seat par excellence of the necrotic foci. The trabeculae had a hyaline appearance and the arteries were dilated. Haemorrhage into the pulp was a constant feature. Plague bacilli were numerous throughout the pulp and might occur inside the nodes. Babes and Livadite (1897) recorded in guinea-pigs and mice the presence of large numbers of giant-cells of megakaryocyte type, especially in the vicinity of the necrotic areas. Blood-corpuscle-containing cells and pigment cells were scarce in spite of extensive pulp haemorrhages.

Albrecht and Gohn (1900) found a great similarity in the main splenic lesions in experimental rat plague by whatever method the inoculation was performed. The nodes were as a rule much diminished and necrotic pulp-foci were of constant occurrence.

Sata (1900) gives a fairly detailed description of the histology of experimental rat plague and lays great stress on the variations met with as regards the distribution of plague bacilli in the organs. Subcapsular haemorrhage was a frequent feature in the spleen and bacilli might be very numerous, very scarce or not demonstrable at all in sections.

Liver. Van der Stricht observed areas of fatty degeneration of the liver cells and also a fine vacuolated condition of the liver cell protoplasm. In one animal small necrotic areas due to capillary emboli were noted.

Honl noted focal necroses surrounded by a zone of leucocytes. Groups of bacilli arranged in zoogloeal masses were present in these foci. Babes and Livadite recorded the presence in the liver capillaries of "Riesenzellen mit gelappten Kern" which have been already alluded to.

Fatty degeneration of the liver cells was noted both by Albrecht and Gohn and Sata. The latter also demonstrated fibrin in the larger vessels. The occurrence of bacilli in the liver capillaries was found to be a very variable factor.

The material of the following six cases was obtained from rats inoculated by the cutaneous method in Bombay. For Rat No. VII of chronic experimental plague I am indebted to Capt. S. R. Douglas.

Rat I. *Spleen* :—Serous endothelium swollen, capsule oedematous with effusion of red cells into it. The endothelial cells of serosa contained red cell débris. There was marked subcapsular haemorrhage.—Nodes : The lymphoid tissue was increased in amount. At the periphery of each node was a circular zone of necrotic cells with bacillary clumps in the neighbourhood. Near the margin of one node was a typical Langhans' giant-cell. The appearance of this cell in five serial sections is indicated in Plate VIII, Fig. 6. In one cross section the constituent nuclei are seen to fill practically the whole cell leaving only a faint rim of protoplasm. The pulp was much congested and numerous zoogloeal bacillary masses were noted. Plasma cells were abundant. The pulp cells showed a slight degree of karyorrhexis. No bacilli were seen in the nodes. *Liver* :—The liver cells were generally healthy apart from the necrotic areas which were numerous. Each focus contained small heaps of bacilli and a good deal of haemorrhage was present at the periphery. Endothelial catarrh of the capillaries was a marked feature. Bacilli were distributed in clumps and many of them were noted inside phagocytic endothelial cells. Fibrin was also observed in the capillaries, along with numerous red cells.

Rat II. *Spleen* :—Marked catarrh of the serous endothelium with subcapsular haemorrhage. The lymphoid tissue was increased and irregularly distributed. Numerous actively dividing large mononuclear endothelial cells were observed in the nodes. A zone of karyorrhectic pulp cells with detritus forms a sort of capsule to each node. Bacilli occurred in clumps surrounded occasionally by necrotic pulp cells. Pyknotic plasma cells were very abundant. In the haemorrhagic areas, fibrin was present in large amount and blood-corpuscle-containing cells were numerous. *Liver* :—Liver cells were not fatty. Marked subcapsular haemorrhage. Necroses were few and small and most of them contained bacillary clumps, surrounded by karyorrhectic nuclei. Bacilli were very numerous in the capillaries and the endothelial cells frequently contained ingested bacilli.

Rat III. *Spleen* :—Capsule swollen and subcapsular haemorrhage. The malpighian bodies were swollen but regular in contour. Round each was a zone of thickened reticulum containing few cells. For the first time, a node was seen whose central artery contained a fibrin thrombus with bacilli. In the pulp, enormous numbers of bacilli were present along with red cells and fibrin. Polynuclear cells, pyknotic plasma cells and endothelial cells were abundant. Haemosiderin cells also occurred frequently. *Liver* :—The liver cells were markedly fatty. Their nuclei also frequently presented karyorrhexis. Necroses were fairly numerous and often of large size. Capillaries containing bacillary emboli were noted in these foci. In the capillaries of the healthy liver substance were enormous numbers of bacilli generally clinging to the walls of the vessels. Round the portal vessels were lymphoid and plasma cell infiltration.

Rat IV. *Spleen* :—Large numbers of bacilli surrounded each node but in only one case were bacilli seen in the follicular artery. The large mononuclear endothelioid cells were abundant in the node and showed signs of active division. Also small necrotic areas containing large endothelial phagocytes and detritus sometimes appeared inside the node. The pulp showed extensive haemorrhages with polynuclear infiltrates and haemosiderin cells. No necrotic foci were present but here and there were small areas in which the cell-nuclei were vesicular. *Liver* :—The liver cells were fatty. Only one or two minute necroses were seen each containing bacilli in its central capillary with effusion of red cells at the periphery. The capillaries throughout the liver substance were quite filled with bacilli and many polynuclear cells were present. Colonies of plasma cells were seen in the sheaths of the large portal vessels in the Glisson's space.

Rat V. *Spleen* :—Capsule thickened, trabeculae increased, and marked subcapsular haemorrhage. The nodes were greatly diminished and irregular in contour. They contained numerous large mononuclear cells of endothelioid type, many of which were dividing. The spleen pulp was greatly congested and bacilli occurred in swarms especially in the large trabecular sinuses. Polynuclear cells, haemosiderin pigment cells and blood-corpuscle-containing cells were abundant in the areas of red cell extravasation. Plasma cells were very numerous and were in active division. No actual pulp necroses were observed. *Liver* :—Only a very few minute necrotic foci were observed in the subcapsular region, involving individual liver cells or a few adjacent ones. Bacilli were also most numerous in this region, lying in clumps in the capillary vessels. The intraacinar capillaries throughout the organ were filled with red cells, infiltrating cells of polynuclear type, and catarrhal endothelial cells.

Rat VI. *Spleen* :—Marked subcapsular haemorrhage and great reduction in the amount of lymphoid tissue. Many of the nodes showed a hyaline thrombosis of the central artery, the lymphoid cells in the neighbourhood being karyorrhectic.

Many endothelial phagocytic cells were present, containing nuclear detritus in their interior. A perinodal zone of karyorrhectic pulp cells was a conspicuous feature. Vesicular epithelioid cells and plasma cells invariably bounded this zone. The pulp was greatly congested with extravasated red cells and polynuclear leucocytes. Bacilli occurred in enormous numbers. Haemosiderin cells and megakaryocytes were also very abundant. *Liver* :—Necroses were few in number. Bacilli were generally to be found in the central capillaries of the focus with effused red cells at the periphery. The large portal sinuses and the intraacinar capillaries throughout the organ were extremely congested and contained enormous numbers of bacilli and polynuclear leucocytes.

Rat VII. *Chronic experimental rat plague.*

The rats in which these chronic plague lesions were found had been inoculated with virulent plague ten days after a partial immunisation with plague vaccine. Those which survived were killed on the eleventh day following the inoculation with the virulent culture.

Protocol:—Spleen much enlarged and contained large grayish caseous-looking areas.—Liver also showed a few grayish nodules.

Spleen :—Microscopical examination (Unna-Pappenheim's stain). No differentiation of the splenic tissue into nodes and pulp was possible. In fact the organ was transformed into a veritable plasma-cell granuloma with abscesses interspersed here and there. A large clump of degenerated bacilli occupied the centre of each necrotic area and all around were broken down polynuclear cells. Bounding this zone of degenerated cells was a band of epithelioid cells, spindle cells and numerous giant-cells of tubercular type. Megakaryocytes also appeared in this zone. Enclosing the whole was a barricade of plasma cells in active division, and transition forms were readily demonstrable between these latter cells and the spindle cells from which the granulation zone surrounding the abscess was being developed (see Plate VIII, Fig. 3). The presence of giant-cells of Langhans' type was especially interesting as confirming the view that the small agglomerations of nuclei above referred to in some of the cases of spontaneous plague were really developing giant-cells. Fully formed cells of this type have been already noted in the liver of Rat IV, Part I. *Liver*:—A section through one of the small subcapsular nodules showed that nothing remained of the original abscess. The nodule consisted solely of spindle cells and fine connective tissue fibres with a boundary zone of actively proliferating plasma cells.

Survey of the experimental cases.

The changes met with in the experimental cases present far more points of resemblance with those of group I of the spontaneous cases than with those of group II.

Extreme bacteriaemia is the rule and the infiltrating cells are found to belong mainly to the polynuclear type. In the spontaneous cases the latter feature was not so prominent.

Focal necroses of the liver cells were invariably scanty. The vessels were all greatly congested and contained large deposits of fibrin.

In the spleen, a noteworthy feature in two cases was the occurrence of thrombosis of the follicular artery accompanied by bacillary incursion and the production of karyorrhectic changes in the nodal cells. Giant-cell formation (of Langhans' type) had evidently not proceeded to any extent, but the discovery of a typical cell of this nature at the periphery of one follicle and bordering a necrotic patch shows that, even in acute experimental cases, the tendency to giant-cell development is at least not in entire abeyance.

The importance of Rat VII, as showing the later stages of the pathological processes already at work in the more acute cases, has been sufficiently alluded to at the close of the histological summary.

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TYPHOID CARRIERS.

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During the past two years the important question of "chronic typhoid carriers" has received a large amount of attention at the hands of German hygienists and bacteriologists.

Hitherto the importance of the subject from the point of view of public health has been insufficiently realized in this country. Recently an American worker has recorded one very interesting case to which reference will be made later. It is not proposed here to discuss in detail the various published cases, which after all present so many points of similarity. It will suffice to refer briefly to the more important articles, after which we shall record our own experiences in this matter.

German Experiences.

For a large part of our knowledge of the subject we are indebted to hygienists in Strassburg. The now classical case of the Strassburg female baker was described by Kayser in 1906. The facts of the case were briefly these: It was observed that practically every new employee in the bakery became sooner or later seriously ill with intestinal typhoid-like symptoms. In two persons the disease proved fatal. The faeces of the woman who owned the bakery and who had had typhoid fever ten years previously, were found to contain typhoid bacilli in large numbers. Her serum also agglutinated the *Bacillus typhosus* up to 1 in 100. Kayser (1906) was also able to isolate another carrier in the person of a female engaged in the milk trade. She was apparently responsible for a small typhoid epidemic due to infected milk, in which 17 cases were involved, with two fatalities.

Further, several epidemics in a household were traced to a female who had had typhoid fever complicated with jaundice thirty years previously. In all, twelve carriers were found (9 female and 3 male). The ratio (3 to 1) probably also expresses the incidence of gall-stone disease in the female and male respectively. Klinger (1906), in an examination of the stools of 482 cases of typhoid fever, found that 27, or 13.1 per cent., harboured the bacilli; of these 27, however, 8 only continued to harbour the bacilli longer than six weeks after the temperature had become normal, so that altogether 1.7 per cent. became chronic carriers. These cases were followed up for varying periods up to eleven months. Klinger places in a separate group a series of 11 cases (6 male and 5 female), who were found to pass typhoid bacilli in the stools for a short time only. These people had not had typhoid fever, but had been closely associated with typhoid patients. These temporary harbourers of the bacillus, or "acute typhoid carriers," he does not consider to be relatively so dangerous as the chronic or persistent carriers. It seems unjustifiable to dogmatize on this point, as the passage of bacilli in the stools probably takes place intermittently, and repeated examination at intervals would be required to settle this question.

Lunatic asylums appear to yield a considerable number of the cases recorded; and, as our experience has been obtained with material from this source, it may be well to summarize the chief evidence.

In the course of an investigation into the causes leading to a succession of typhoid epidemics which had occurred in an asylum containing 250 patients, Nieter and Liefmann (1906) were able to isolate seven typhoid carriers. They had commenced with an examination of the stools of patients who had recovered from typhoid fever, but none of these were found to harbour the typhoid bacillus. A certain patient, however, happened to be suffering from dysentery, and in her stools typhoid bacilli were found, along with dysentery bacilli of type "Flexner." In the faeces of another patient who had the characteristic symptoms of dysentery, typhoid bacilli were found, but no dysentery bacilli. After death typhoid bacilli were found, not only in the intestine, but also in the bile. The blood of this patient also agglutinated *B. typhosus* up to 1 in 50, and the *B. dysenteriae* (Flexner) up to 1 in 100. Old typhoid cicatrices were found in the small intestine, while the large intestine showed chronic catarrh.

Probably the infection of these dysentery cases with the *Bacillus typhosus* was due to the fact that, owing to lack of room, the typhoid and dysentery cases were not isolated in separate buildings. The transference of infection from one patient to another is readily conceivable when the filthy habits of many of these cases are remembered.

Further notes on the conditions prevailing in this asylum were recently recorded by Nieter (1907). The asylum contained altogether about 900 females, and the previous 7 cases had been discovered in one building containing 250 female inmates. In addition to these 7 cases, 4 others were found, and it was hoped that with the isolation of these no more outbreaks would arise. However, during the course of December, 1906, and January, 1907, 3 cases of typhoid fever occurred among female attendants. In the room in which these attendants had served was found another carrier. This female harboured the paratyphoid bacillus (type B) in addition to the *Bacillus typhosus.*

Four or five months later an outbreak occurred in another department. This led to the discovery of yet another chronic carrier who had had typhoid fever two years previously. Altogether in this asylum 13 cases were brought to light.

The experience of Friedel (1907) is especially interesting, as the outbreaks occurring in a similar institution were clearly traced by him to an imbecile patient who did work in the kitchen department. This person had never suffered from typhoid fever. It is important to note that her work in the kitchen consisted chiefly in the preparation of various salads.

In an asylum at Hördt as described by Klinger, two carriers were found and isolated. It happened that in the typhoid-infected building there was a washerwoman who had suffered from typhoid fever one year previously. Her stools were examined three times, and found to be free of typhoid bacilli (February and March, 1905). However, in September, 1905, other cases broke out in her department, and she was now found to be a carrier. Her serum agglutinated the *Bacillus typhosus* up to 1 in 1,000.

Other carrier cases isolated in asylums at Saargemünd and Klingemünster are mentioned by Nieter. The American case described recently by Soper (1907) of New York concerned a female cook, in apparently perfect health, who, so far as her history could be traced, had in the course of five years been responsible for the occurrence of typhoid epidemics in seven different households. Her stools were found to contain typhoid bacilli in large numbers, and her serum gave a positive Widal reaction.

CAUSE OF THE PRESENT INVESTIGATION.

A certain lunatic asylum in Scotland, containing at the present time 92 male and 53 female patients, 8 male and 6 female attendants, and 4 female servants, has since 1893 been visited by a succession of small outbreaks of typhoid fever. Similar outbreaks have occurred during the same time in a smaller detached institution situated about five minutes' walk from the main building. Female patients only, to the number of 32, occupy at present this smaller asylum. Prior to the adoption of the Notification Act in 1891 no authentic records of typhoid fever in this institution are available.

Thirty-one cases in all have 'occurred, and 24 of these have been females. Nine cases have proved fatal, giving a fatality rate of 29 per cent. Included in the accompanying table are 4 cases among the attendant staff (3 female and 1 male). It will be observed from the table that the cases occurred at no special time of the year, but that in any one year the notified cases followed each other as a rule very closely. During 1894, 1898, 1902, and 1905, no cases were notified.

As might be expected, the mysterious cropping up of typhoid cases has been a source of great anxiety to the authorities, who have done everything in their power to make the sanitary arrangements of the institution as complete as possible. Repeated analyses of the water supply, both chemically and bacteriologically, have thrown no light on the etiology of the outbreaks. The sewage is conveyed direct to the sea. The dairy arrangements are in excellent order. The same water supply is enjoyed by several families residing on a farm near the source of the supply, but at no time have cases of typhoid occurred among them. These and other considerations suggested that the source of the infection was possibly to be found among the patients themselves, and in the light on recent German work on the occurrence of typhoid carriers, one of us (A. L.) proceeded to investigate the whole question from this point of view. As three-fourths of the cases had occurred among the female inmates, his attention was confined solely to these. In February of this year a start was made by testing the Widal reaction in a group of 43 female patients. The serum of 6 cases, 2 of whom had had typhoid fever previously (in 1903 and 1904 respectively) gave a well-marked reaction. In the course of this investigation, however, A. L. himself contracted an attack of typhoid fever, and nothing

Table Showing the Incidence of Typhoid Fever in Successive Years since 1893, when the First Case was Notified.

Year.	Total Number of Cases.	Month of Notification.	Main Asylum.	Small Asylum.
1893	2	March, July	2	0
1894	0	——	0	0
1895	6	July (2), August (3), December (1)	1	5
1896	2	April, May	2	0
1897	1	November	0	1
1898	0	——	0	0
1899	4	August (1), November (3)	4	0
1900	3	January, June, August	1	2
1901	2	February, May	2	0
1902	0	——	0	0
1903	4	February, March, April, October	4	0
1904	3	June, July, August	3	0
1905	0	——	0	0
1906	1	April	1	0
1907	3	February, April, October	2	1

further was done in the matter till September, when it was determined to make an exhaustive bacteriological examination of the stools of all the female patients in the asylum. The samples were forwarded to one of us (J. C. G. L.) at the Lister Institute, London.

Bacteriological Investigation.

The samples were received in sterile bottles, great care being taken to avoid any admixture of urine, as the latter markedly inhibits the growth of intestinal organisms on the plates. From each sample two separate loops were taken and emulsified in two broth tubes. Two plates of bile salt lactose-neutral-red agar (MacConkey's medium) were spread with a loopful from each broth tube, four plates in all being prepared from each sample of faeces.

On the following day the colourless colonies were picked off and inoculated in mannite-peptone water. If gas production occurred (as in the great majority of the cases), no further consideration was necessary. Where acid production only occurred, a further series of sugar tubes were inoculated (glucose, lactose, cane sugar, and dulcite), also litmus, milk, and gelatine. Indol was tested for by Ehrlich's method on the third day. Agglutination tests were made with an antityphoid horse serum, also with serum from positive cases of typhoid fever.

From the main asylum fifty-seven samples of faeces from female patients were examined. One patient, G., was found to be passing typhoid bacilli in enormous numbers in the faeces. In fact, the plates contained typhoid colonies to the almost complete exclusion of *B. coli* and other lactose fermenters. This patient, G., has been in the asylum since 1896. She is 35 years of age, suffers from dementia, and there is no record of her having had typhoid fever. Her personal habits are exceedingly filthy, and she has never been employed in the laundry or kitchen. Her serum agglutinated the *Bacillus typhosus* up to 1 in 200. (Time, 1½ hours at room temperature.)

For this test the laboratory strain was employed. Curiously enough, her own strain was agglutinated very slowly by G.'s serum, but was agglutinated by antityphoid horse serum in dilution of 1 in 8,000. The G. strain had also considerable virulence for the guinea-pig; 2 c.cm. of a twelve-hour bouillon culture inoculated intraperitoneally caused death within twenty-four hours.

The patient G. was isolated on September 24th and treated with intestinal antiseptics. On October 12th samples of faeces and urine of G. were received. Typhoid bacilli were not found in either. On October 21st the *B. typhosus* was readily isolated from the faeces, and a fourth sample received on November 29th was also positive. The urine remained negative.

With the isolation of G., it was hoped that no more cases of typhoid fever would recur, but this did not prove to be the case. Towards the end of October a case of typhoid fever occurred in the small asylum, and accordingly we proceeded to examine the faeces of all the female patients systematically (34 in number). Another carrier was thus brought to light. This patient, M C., is about 60 years old. She had an attack of typhoid fever in 1895, and has been continuously in the small asylum since that time. She is able to walk about and do a little work, but she has never been employed in the kitchen. Her habits are cleanly, and she has occasionally assisted in the laundry. It has to be noted that though there are two separate blocks, they are really one, from the extent of communication that goes on between them. The dinners are cooked at the main asylum and brought over to the smaller block. The clothes are washed also at the main asylum, but patients from the small asylum would occasionally assist. As in G.'s case, the plates of M'C. showed almost pure typhoid to the exclusion of lactose fermenters.

The strain obtained from M'C. was agglutinated by antityphoid serum in dilution of 1 in 7,000. The serum agglutinated the laboratory strain, her own strain, and the G. strain up to 1 in 200. The M'C. strain had also the same degree of virulence for the guinea-pig as the G. strain. On November 29th, the *Bacillus typhosus* was again isolated from the stools of M'C. but not from the urine.

So far the blood has not been examined for typhoid bacilli in either G. or M'C.

All the females in both buildings had now been gone over, but it was felt advisable to repeat the examination in some of them, especially those who had suffered from typhoid fever in recent years.

A third carrier negative on the previous occasion was thus revealed.

This patient, C., is about 30 years old and had typhoid fever in 1904. She is not of dirty habits, and has never been employed in kitchen or laundry. The plates prepared from C. showed very few colonies, and in this respect differed from those of G. and M'C. The serum gives a marked Widal reaction, and the strain obtained from the stools is agglutinated by antityphoid serum in dilution of 1 in 7,000.

Up to the present time, therefore, we have succeeded in isolating three typhoid carriers out of a total of ninety females examined. The male patients have so far not been investigated, but we are at present considering this step.

Prophylactic Measures.

We shall conclude with some observations as to the treatment adopted in these cases. In addition to isolation and disinfection of excreta, so-called intestinal and urinary antiseptics have been employed in our cases, but it is not at all likely that they will do the slightest good. Other workers have recorded quite negative results with milk diet, chloroform, hepatic cholagogues, salol, etc.

It seems probable that in these cases the bacilli vegetate in the gall bladder, from which they are intermittently ejected into the intestine. An interesting case, showing the importance of the gall bladder as the seat of vegetation of the *Bacillus typhosus*, is recorded by Levy and Kayser (1907). A female asylum patient who had been isolated as a typhoid carrier on October 17th, 1905, died on October 18th, 1906, after a short illness, in which fever, pulmonary trouble, and stupor were marked symptoms. At the necropsy, hypostatic pneumonia was found, the spleen was slightly enlarged, and a gall stone was present in the fundus of the gall bladder. Typhoid bacilli were isolated from the liver, spleen, bile, gall bladder wall, and interior of gall stone. In view of the general distribution of the typhoid bacillus in this case, the authors conclude that death was due to an autoinfection from the gall bladder, where the bacillus had so far led a saprophytic existence.

Dehler (1907) has actually performed cholecystostomy and drainage of the gall bladder in two asylum carrier cases, though in neither were there symptoms pointing to gall-bladder disease.

Numerous examinations of the stools were made after the operations, and it was found that in a few months the typhoid bacilli ceased to appear in the faeces, and the patient's serum failed to agglutinate the typhoid bacillus.

It cannot, however, be expected that this form of treatment will become popular, at least where otherwise healthy carriers are concerned.

The appropriate treatment of these cases presents many difficulties, but one conclusion is clear—that a person who is found to be a typhoid carrier should be kept constantly under bacteriological supervision. Perhaps also many typhoid epidemics would be avoided if the excreta of recovered typhoid cases (especially female cases) were examined systematically (say once a month) up to six months after recovery.

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THE PARA-DIMETHYL-AMIDO-BENZALDEHYDE TEST FOR INDOLE[1].

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(Three Charts.)

This indole reaction, first described by Ehrlich, and used by him as a urinary test, has been applied by Böhme in determining the presence of indole in bacterial cultures. Böhme claims for this test that it is more delicate and more exact than the usual nitrite and sulphuric acid test, and it has given such good and constant results in this laboratory, that the following facts and figures may be given in confirmation of Böhme's claim.

The test, as recommended by Böhme, consists of two solutions made up as follows:—

Solution 1.	Para-dimethyl-amido-benzaldehyde ...	4 parts.
	Absolute Alcohol	380 „
	Concentrated Hydrochloric Acid ...	80 „

Solution 2. Potassium persulphate in saturated watery solution. To about 10 c.c. of the broth culture of the organism add 5 c.c. of solution 1, and then 5 c.c. of solution 2, shake the mixture and the presence of indole is indicated by the appearance, in a very short time, of a red colour, which gradually becomes darker on standing

This test can not only be used qualitatively for detecting the presence of indole, but it can also be used quantitatively for estimating the amount of indole produced by the micro-organism.

As a Qualitative test. I think most observers will agree that the nitrite and sulphuric acid test is not always satisfactory, and there is often great doubt as to whether a slight reaction really means the production of indole by the micro-organism. The formation of indole, however, can be accurately determined by distillation and this fact was used as a confirmation in comparing the two tests, which, for the sake of brevity, we will now call the "old" (nitrite and sulphuric acid) and the "new" (para-dimethyl-amido-benzaldehyde).

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[1] The usual spelling "indol" is here altered to "indole" in accordance with a rule laid down by the Chemical Society whereby the termination "ol" is in future to be used to indicate substances containing an OH group.—Ed.

Fifteen different micro-organisms were inoculated into peptone beef broth, and, at various intervals of time, were tested for indole, both tests being used. The results obtained are shown in the following table:

	24 hours' culture		3 days' culture		5 days' culture		12 days' culture	
Micro-organism	New test	Old test	New test	Old test	New test	Old test	New test	Old test
B. enteritidis (Gaertner)	0	0	0	+	0	+	0	trace
B. typhi murium	0	trace	0	+	0	trace	0	+
B. psittacosis	0	trace	0	+	0	+	0	trace
B. Hanstedt (Fisher)	0	0	0	+	0	+	0	+
B. cloacae	0	+	0	+	0	+	0	+
B. of epidemic jaundice	+	+	+	+	+	+	+	+
B. acidi lactici	+	+	+	+	+	+	+	+
B. Hog cholera	0	trace	0	+	0	+	0	trace
B. Abel	0	+	0	+	0	+	0	+
B. coli communis	+	+	+	+	+	+	+	+
B. dysenteriae (Flexner-Gray)	+	trace	+	+	+	+	+	+
B. pyogenes fetidus	+	trace	+	+	+	+	+	+
B. typhosus	0	trace	0	trace	0	+	0	+
B. paratyphoid A	0	trace	0	+	0	+	0	+
B. paratyphoid B	0	trace	0	+	0	+	0	+

According to the new test only five micro-organisms in the above series produced indole, namely, *B. of epidemic jaundice, B. acidi lactici, B. coli, B. dysenteriae* (Flexner-Gray) and *B. pyogenes fetidus.* Accordingly, large quantities of peptone beef broth, 50 c.c. to 100 c.c., were inoculated with these micro-organisms, incubated at 37° C. for some days, and then distilled. The distillate was tested for indole as before.

			Distillate	
Micro-organism	Amount of culture	Time grown at 37° C.	New test	Old test
B. coli communis	100 c.c.	9 days	+	+
B. dysenteriae (Flexner-Gray)	100	8	+	+
B. pyogenes fetidus	50	9	+	+
B. acidi lactici	50	9	+	+
B. of epidemic jaundice	100	5	+	+

This proved that these five micro-organisms, which always gave a constant reaction with the new test, were real indole producers, and the question next arose as to whether the positive reaction given with the old test by the other micro-organisms denoted the presence of indole. The following were therefore also distilled and the distillate tested.

Micro-organism	Amount of culture	Time grown at 37° C.	Distillate New test	Distillate Old test
B. enteritidis (Gaertner)	100 c.c.	8 days	0	0
B. Abel	100	8	0	0
B. cloacae	100	6	0	0
B. paratyphoid A	100	7	0	0
B. paratyphoid B	100	7	0	0
B. typhosus	100	8	0	0

When, in addition to these results, we find that the para-dimethyl-amido-benzaldehyde gives a definite pink colour with ·0001 milligramme of indole, whereas the nitrite and sulphuric acid test is barely perceptible with ·0005 milligramme of the substance and fails entirely with ·0002 milligramme, we must conclude that the para-dimethyl-amido-benzaldehyde test is both more accurate and more sensitive.

As a Quantitative test. Herter and Foster, and Peckham, have described methods for the quantitative determination of indole, the former observers using B. napthaquinone-sodium-monosulphonate and the latter sulphuric acid and nitrite. I have endeavoured to ascertain whether para-dimethyl-amido-benzaldehyde can also be used colorimetrically in the quantitative estimation of indole, and have found the following method the most satisfactory.

A standard solution of indole is required, and is obtained by dissolving ·05 gramme of indole in 5 c.c. of absolute alcohol and then adding distilled water to 500 c.c. Five c.c. of the distillate to be tested for indole are then taken (having added 1 c.c. of absolute alcohol for every 100 c.c. of the distillate), added to a Nessler's tube and made up to 50 c.c. with distilled water. To this add 2 c.c. of the para-dimethyl-amido-benzaldehyde solution and 2 c.c. of the potassium persulphate solution and thoroughly mix. As before, the presence of indole is indicated by the appearance of a red colour. Varying quantities of the standard solution are then similarly treated in a series of Nessler's tubes until the requisite colour is obtained, when the amount of indole in the distillate can be calculated. The mixtures should be allowed to stand for one hour before matching the colours, as in some tubes the colour sometimes develops more quickly than in others in which there is more indole and which eventually gives a darker colour. A series of Nessler's tubes, containing, say, ·2, ·3, ·4 and ·5 c.c. of the standard solution of indole give quite distinct grades of colour, so that in two solutions, a difference of ·01 milligramme of indole can be accurately determined.

In testing this method the following three series of experiments were done, in order to show the variations in the amount of indole, produced by the same micro-organism, grown for different periods of time.

Seven flasks, each containing 100 c.c. of peptone beef broth were inoculated with 1 loopful of an agar culture of *Bacillus coli*, incubated at 37° C. and distilled on succeeding days. The distillates, tested for indole, gave the following results:

Distillate after 1 days' growth = 0·86 milligrammes of indole per 100 c.c.
" " 2 " " = 1·20 " " " "
" " 3 " " = 1·80 " " " "
" " 4 " " = 2·00 " " " "
" " 5 " " = 3·80 " " " "
" " 6 " " = 3·60 " " " "
" " 7 " " = 2·40 " " " "

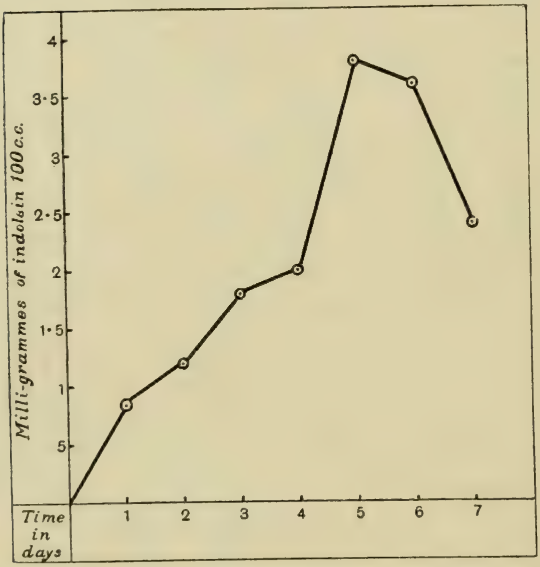

CHART I. Showing the amount of indole present on 7 consecutive days during the cultivation of *Bacillus coli* at 37° C.

In this series the indole production gradually rose to a maximum on the fifth day, with a slight falling off on the sixth day, and a still greater falling off on the seventh day. It was considered advisable to repeat this experiment with *Bacillus coli*, but to carry it on for a longer time. Another series of 100 c.c. flasks of peptone beef broth were inoculated with 5 drops, with a sterilized pipette, from a 24 hours' broth culture of *Bacillus coli*, this being considered more accurate than the standard loop. As before these flasks were incubated at 37° C., the first distillation carried out after four days' growth and the last 23 days after inoculation. The distillates gave the following figures:

Distillate after 4 days' growth = 1·20 milligrammes of indole per 100 c.c.
" " 5 " " = 1·30 " " " "
" " 6 " " = 1·80 " " " "
" " 7 " " = 1·60 " " " "
" " 10 " " = 1·80 " " " "
" " 11 " " = 2·00 " " " "
" " 14 " " = 2·90 " " " "
" " 23 " " = 1·80 " " " "

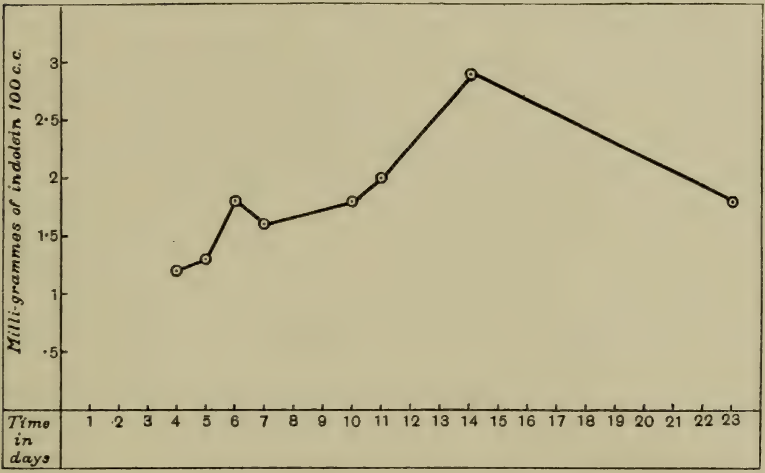

Chart II. Showing the amount of indole present at various stages during the cultivation of *Bacillus coli* for 23 days at 37° C.

In this series the indole production was very much slower, probably on account of the different method of inoculating the flasks. The indole production rose from the fourth to the sixth day, fell a little on the seventh and then rose to a maximum on the fourteenth day.

It was then considered advisable to estimate the indole production by another micro-organism, and cholera was chosen, because it was found on estimation to produce more indole than any other of the indole-producing organisms we had tested. This choice was rather unfortunate, as the nitrite produced by the cholera vibrio was sometimes present in the distillate and it was found that the presence of a nitrite has an inhibitory action on the production of the red colour. Nitrites in strong solutions give a yellow colour on the addition of the reagents. In this series, as in the preceding one, five drops of a bouillon culture were used to inoculate the flasks. The first distillation on this occasion, was done early, seven hours after inoculation, and the last flask had been incubating 27 days before it was distilled. The distillates were tested for nitrites.

			Nitrites
Distillate after 7 hours' growth	=0·0	milligrammes of indole per 100 c.c.	0
,, ,, 24 ,, ,,	=0·0	,, ,, ,,	+ + +
,, ,, 48 ,, ,,	=0·0	,, ,, ,,	+ + +
,, ,, 4 days' ,,	=0·7	,, ,, ,,	+ + +
,, ,, 6 ,, ,,	=0·8	,, ,, ,,	0
,, ,, 9 ,, ,,	=2·0	,, ,, ,,	+ +
,, ,, 13 ,, ,,	=2·8	,, ,, ,,	+ +
,, ,, 18 ,, ,,	=7·0	,, ,, ,,	0
,, ,, 27 ,, ,,	=4·8	,, ,, ,,	0

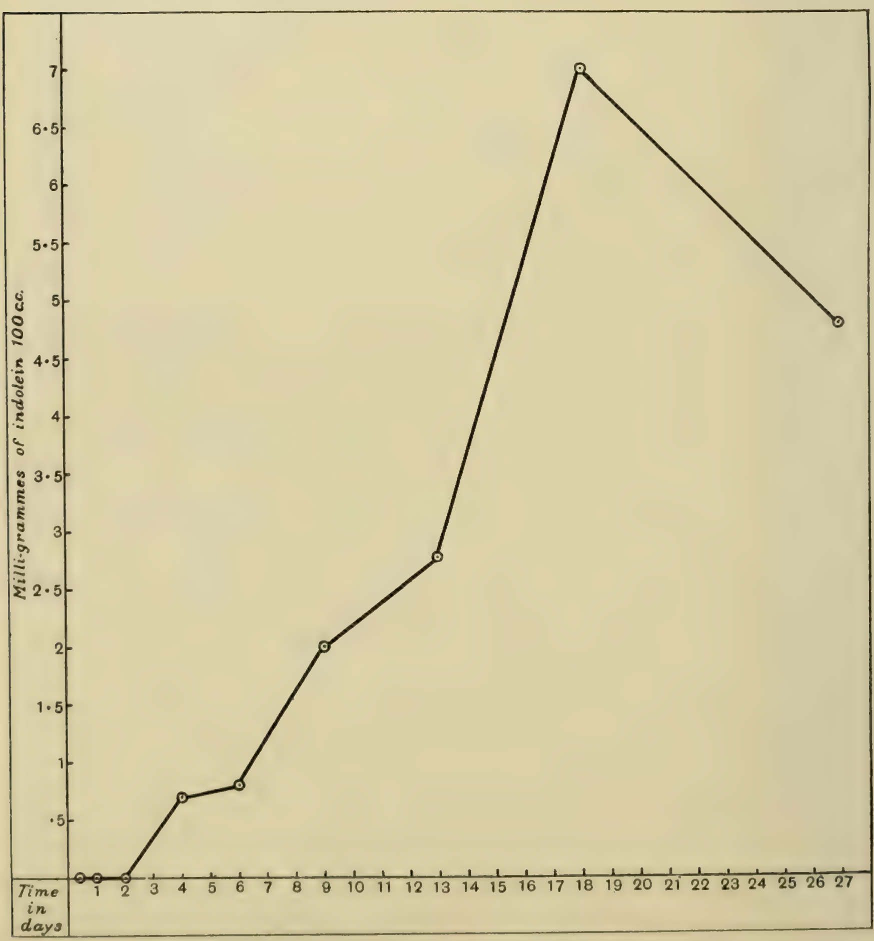

Chart III. Showing the amount of indole present at various stages during the cultivation of the *cholera vibrio* for 27 days at 37° C.

This completed the series and these three curves of indole production are exceedingly interesting, in that they bear a striking resemblance to curves of toxin production. Probably the metabolic processes concerned in the two functions run parallel and certain circumstances which favour toxin formation also favour indole production. They are sufficient, at any rate, to demonstrate the use of this test in the quantitative estimation of indole.

It can also be demonstrated that the presence of lactose and glucose inhibit the production of indole by bacteria. *Bacillus coli*, grown at 37° C. for five days in 100 c.c. peptone beef broth containing 2 % lactose, and then distilled, failed to show the least trace of indole. In a similar experiment, with 2 % glucose in the broth, only a very small quantity of indole was produced. Here again there is a parallelism between indole production and toxin formation, as the latter, in the case of both the Diphtheria and Tetanus bacillus, is also inhibited by the presence of excess of glucose.

Skatole gives with this reagent a violet colour, which, on standing, becomes deep blue (the appearance of the blue colour is hastened by the addition of the persulphate). Only on one occasion have I found skatole in bacterial cultures, and that was in a broth culture of *Bacillus typhosus*, freshly isolated from the blood.

It may be added in conclusion, that, for laboratory purposes, this test for indole is exceedingly useful. There is no doubt about the result, it shows the presence of very small amounts of indole, and it is easily applied. In the routine examination of organisms isolated from water and from milk, we can say quite definitely in 24–48 hours if indole be present, a great saving of time, as it was customary to wait five days before applying the nitrite and sulphuric acid test.

Summary and Conclusions.

1. The high estimate formed by Böhme of the utility of this test has been fully confirmed.

2. Other substances, such as skatole-carboxylic acid, which tended to confuse the results of the older method, do not give an indole-like reaction with this reagent.

3. The test is more delicate, and the time required to ascertain the presence of indole is shortened.

4. The method lends itself to accurate colorimetric quantitative estimations, and the amount of indole present at various stages during the cultivation of certain organisms has been determined.

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THE INFLUENCE OF DILUTION OF SERUM ON ITS OPSONISING POWER.[1]

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(Plates XXXVIII.–XLI.)

Recent contributions by Dean and by Simon, Lamar, and Bispham have drawn attention to the importance of the dilution of the serum on its opsonising power. Dean found that the dilution of fresh, unheated, normal human serum was not accompanied by any fall in the phagocytic power until a quarter concentration had been reached, and he also found that the points corresponding to the $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, and $\frac{1}{32}$ dilutions of normal human serum lay on a parabola which showed that the phagocytosis was proportional to the square root of the serum concentration.

Simon, Lamar, and Bispham, on the other hand, using a different method of estimating phagocytic power, found great variations in the results obtained on the dilution of the sera of normal individuals and of animals of the same species.

In the experiments to be described the ordinary method of Wright and Douglas was used, the diluent employed being normal saline solution. The number of leucocytes counted was never less than fifty, and the slides were so arranged that the counter was quite unaware which slide he was counting.

The following experiments were undertaken to show the effect in such dilution experiments of the injection of large doses of killed bacteria:—

I. Influence of the Dilution of the Normal Serum of the Rabbit and of the Ox on Phagocytosis.

Five different experiments with normal rabbit serum and human leucocytes, the micro-organism used being the *Staphylococcus pyogenes aureus*, gave the following results:—

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[1] Received December 18, 1907.

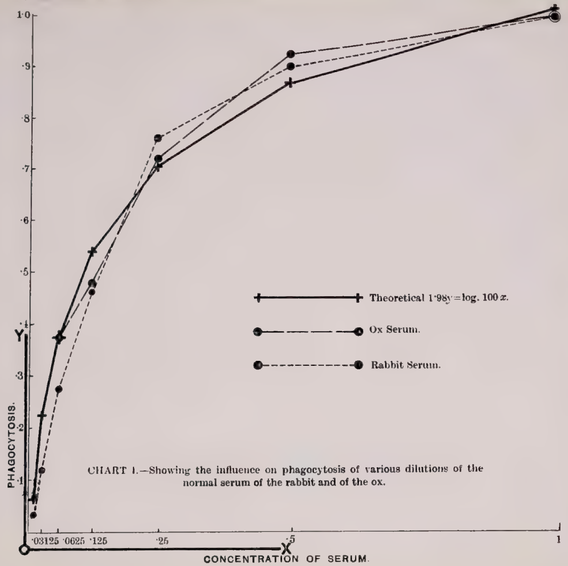

CHART I.—Showing the influence on phagocytosis of various dilutions of the normal serum of the rabbit and of the ox.

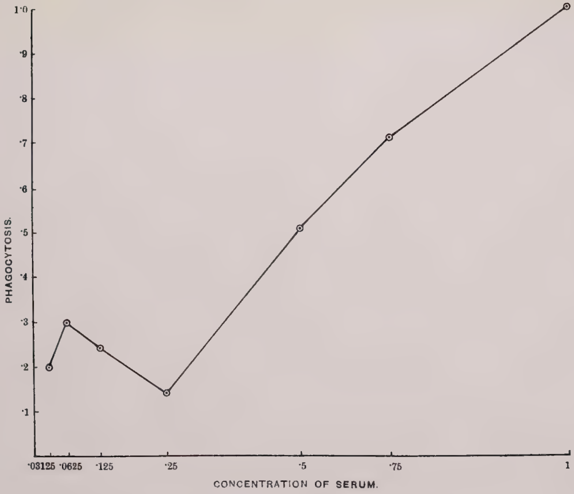

CHART II.—Rabbit E, showing the phagocytosis in the presence of various dilutions of the serum twenty-four hours after intravenous injection with three agar tubes of killed Staphylococcus pyogenes aureus.

		Concentration of Serum.						
		1	0·5	0·25	0·125	0·0625	0 03125	0·015625
Phagocytosis	Rabbit 1 .	1	0·991	0·943	0·761	0·477	0·305	0·186
	,, 2 .	1	1·003	0·953	0·580	0·327	0·176	0·133
	,, 3 .	1	0·894	0·546	0·479	0·290	0·156	...
	,, 4 .	1	0·731	0·717	0·307	0·158	0·081	...
	,, 5 .	1	1·014	0·877	0·566	0·444	0·200	0·012
	Average .	1	0·910	0·773	0·483	0·304	0·153	0·072

Seven different experiments with normal ox serum, using as before human leucocytes and *Staphylococcus pyogenes aureus*, gave the following results :—

		Concentration of Serum.						
		1	0·5	0·25	0·125	0·0625	0·03125	0·015625
Phagocytosis	Ox 1 . .	1	1·006	0·683	0·545	0·369	...	...
	,, 2 . .	1	0·785	0·961	0·684	0·709	...	...
	,, 3 . .	1	0·952	0·458	0·506	0·211	...	...
	,, 4 . .	1	0·956	0·674	0·676	0·325	...	...
	,, 5 . .	1	1·147	0·972	0·289	0·345	...	...
	,, 6 . .	1	0·698	0·701	0·536	0·395	...	...
	,, 7 . .	1	1·020	0·707	0·318	0·383	...	...
	Average .	1	0·937	0·736	0·507	0·391	...	...

The dilution of the normal serum of these two animals evidently differs from the dilution of normal human serum, as there is usually a distinct fall in the phagocytosis on one-half concentration of the serum. The two curves obtained (Plate XXXVIII. Chart I.), with rabbit serum and ox serum, practically coincide, and I am indebted to my colleague, J. C. G. Ledingham, for demonstrating to me that these correspond to a logarithmic curve which satisfies the equation $1{\cdot}98y = \log. 100x$, or, in other words, the phagocytosis is a linear function of the logarithm of the concentration.

In a few preliminary experiments on the dilution of the normal unheated human serum with staphylococci and tubercle bacilli my results were similar to those obtained by Dean, which have been referred to in the beginning of this paper.

II. EFFECT PRODUCED UPON THE NORMAL RABBIT SERUM CURVE BY THE INTRAVENOUS INJECTION OF LARGE DOSES OF KILLED *Staphylococcus pyogenes aureus.*

EXPERIMENT 1.—*Rabbit E.*—This rabbit was injected intravenously with three agar tubes of killed *Staphylococcus pyogenes aureus,* and bled twenty-four hours after inoculation. The phagocytosis obtained on the dilution of the serum gave the following result:—

Concentration of serum .	1	0·75	0·5	0·25	0·125	0·0625	0·03125
Phagocytosis . . .	1	0·71	0·51	0·14	0·24	0·30	0·20

giving the curve shown in Plate XXXVIII. Chart II.

EXPERIMENT 2.—*Rabbit M.*—This rabbit was injected intravenously with five agar tubes of killed *Staphylococcus pyogenes aureus* on 23rd May, and again with three agar tubes on 24th May. It was bled on 25th May, twenty-four hours after the second injection. The opsonic index for *Staphylococcus pyogenes aureus,* compared with normal Rabbit C, was 0·70, and the dilution of the serum gave the following:—

Concentration of serum . .	1	0·75	0·5	0·33	0·25	0·125	0·0625	0·03125
Phagocytosis	1	0·32	0·21	0·21	0·057	0·008	0·059	0·049

giving the curve shown in Plate XXXIX. Chart III.

EXPERIMENT 3.—*Rabbit S.*—This rabbit received intravenously twelve agar tubes of killed *Staphylococcus pyogenes aureus,* and was bled at intervals of one hour, five hours, and twenty-five hours after the injection. The opsonic indices at these intervals were 1·32, 1·37, and 0·71 respectively, the serum of normal Rabbit C being used as the control. The results obtained on the dilution of the serum at these intervals were as follow:—

		Concentration of Serum.							
		1	0·75	0·5	0·33	0·25	0·125	0·0625	0·03125
Phagocytosis	After 1 hour	1	0·73	0·36	0·15	0·18	0·05	0·04	0·08
	,, 5 ,,	1	0·75	0·47	0·51	0·24	0·15	0·07	0·05
	,, 25 ,,	1	0·38	0·49	0·30	0·40	0·12	0·04	0·06

giving the curve seen in Plate XXXIX. Chart IV.

EXPERIMENT 4.—*Rabbit Z.*—This rabbit received intravenously ten agar tubes of killed *Staphylococcus pyogenes aureus,* and was bled twenty hours after injection. The opsonic index, compared with Rabbit C, was 1·36, and the opsonic index for the tubercle bacillus 0·67. Dilution of the serum with both these micro-organisms gave the following figures:—

Concentration of serum . .	1	0·5	0·25	0·125	0·0625	0·03125
Phagocytosis of *Staphylococcus pyogenes aureus* . . .	1	0·37	0·10	0·10	0·09	0·13
Phagocytosis of tubercle bacillus	1	0·82	0·77	0·76	0·57	0·36

giving the curves shown in Plate XL. Chart V.

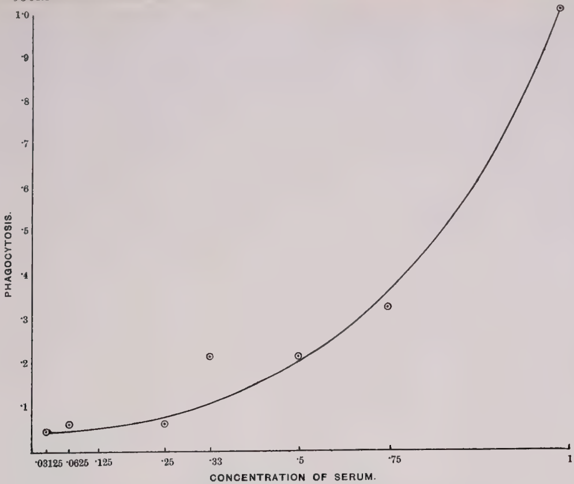

CHART III.—Rabbit M, showing the phagocytosis in the presence of various dilutions of the serum after intravenous injection on May 23 with five agar tubes of killed Staphylococcus pyogenes aureus, and again on May 24 with three agar tubes; bled May 25. Opsonic Index = 0·70.

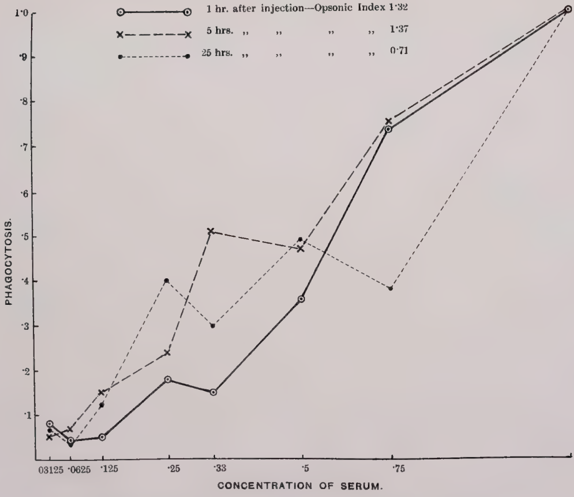

CHART IV.—Rabbit S, showing the phagocytosis in the presence of various dilutions of the serum one hour, five hours, and twenty-five hours after the intravenous injection with twelve agar tubes of killed Staphylococcus pyogenes aureus.

There seems little doubt, from the foregoing experiments, that the injection of the large doses of killed *Staphylococcus pyogenes aureus* has a marked effect on the phagocytic curve of normal serum for that organism. There is a quick and rapid fall in the opsonising power of the serum. This phenomenon appears to be limited to the micro-organism injected, as with the tubercle bacillus a curve corresponding to the normal was obtained. The opsonic index of the undiluted serum gives no indication how the curve will go, some rabbits with a low opsonic index giving a normal curve (Rabbit S before injection gave an opsonic index of 0·56, and Rabbit M before injection gave an opsonic index of 0·70). On the other hand, after the injection of large doses of killed bacteria, some rabbits gave a comparatively high opsonic index; nevertheless, on diluting the serum a marked fall in the phagocytic curve was obtained, and, compared with the normal rabbit curve, the opsonic index varied at each dilution.

III. INFLUENCE OF THE DILUTION OF IMMUNE SERUM UPON PHAGOCYTOSIS.

Rabbit B, having being immunised with numerous subcutaneous injections of killed *Staphylococcus pyogenes aureus*, was bled thirteen days after the last injection, and the phagocytosis on dilution was as follows:—

Concentration of serum . .	1	0·5	0·25	0·125	0·0625	0·03125
Phagocytosis	1	0·61	0·50	0·65	0·52	0·51

It was again bled five days afterwards, *i.e.* eighteen days after the last injection, when the following figures were obtained:—

Concentration of serum	1	0·75	0·5	0·25	0·125	0·0625	0·03125
Phagocytosis . . .	1	0·91	0·79	0·72	0·63	0·84	0·62

The opsonic index on both occasions was less than unity—in the first instance being 0·71, and in the second 0·97, and the curves obtained are shown in Plate XL. Chart VI.

In the experiments on the dilution of the immune serum the most striking phenomenon was the good phagocytosis obtained even on the thirty-second dilution of the serum,—in fact, the phagocytosis did not in the one case fall below 50 per cent. of the maximum, while in the other it never fell below 60 per cent. of the maximum. Compare this with the normal curve, where, on the thirty-second dilution of the serum, the phagocytosis is 25 per cent. of the maximum.

Immune Horse (Rich).—This horse, during a long period of time (about two years), immunised with the staphylococcus, was also tested, and the dilution of the serum gave the following:—

Concentration of serum	1·0	0·75	0·5	0·33	0·25	0·125	0·0625	0·03125
Phagocytosis . . .	0·42	0·81	1·0	0·66	0·52	0·46	0·30	0·13

The opsonic index compared with normal horse (Clipper) serum was so low, 0·51, and the enormous increase in the opsonising power on the half concentration of the serum was so striking, that it seemed desirable to repeat the experiment. Accordingly this horse was again bled one week afterwards, and on this occasion the following figures were obtained:—

Concentration of serum .	1·0	0·75	0·5	0·33	0·25	0·125	0·0625	0·03125
Phagocytosis	0·52	0·51	0·62	0·87	1·0	0·37	0·15	0·23

The opsonic index, compared with normal (cart-horse) serum, was 0·62, and on this occasion the maximum phagocytosis was obtained on the $\frac{1}{4}$ dilution of the serum. The curves are shown in Plate XLI. Chart VII.

Another peculiarity about this immune serum was that it contained no demonstrable thermostable immune substance, nor, as the following figures will show, could it be complemented by the addition of fresh normal serum. In both these respects it differed from the serum of the immune Rabbit B.

Heated immune serum (Rich) + normal unheated serum (Clipper) + leucocytes + cocci = 9·72
,, ,, + ,, ,, (Horse 3) ,, = 9·54
Heated normal serum (Clipper) + normal unheated serum (Clipper) ,, = 10·98
,, ,, ,, + ,, ,, (Horse 3) ,, = 10·80

Heated Immune Serum (*Rabbit B*).—As it seemed desirable to determine the effect upon phagocytosis of the dilution of the thermostable substance present in immune sera, the serum of Rabbit B was heated to 60° C. for about twenty-five minutes, and the following results were obtained on dilution :—

Concentration of serum .	1·0	0·75	0·5	0·25	0·125	0·0625	0·03125
Phagocytosis . . .	0·61	0·73	1·0	0·62	0·84	0·77	0·56

giving the curve seen in Plate XLI. Chart VIII.

This demonstrates that the thermostable substance in immune serum stands dilution very well, and it is probably the presence of this substance in unheated immune serum which causes the marked phagocytosis in the high dilutions of that serum. The anomalous curves obtained with the serum of the immune horse (Rich), however, are difficult to explain, and the absence of thermostable substance is also interesting, as the serum of this horse in the early stage of immunisation was proved to contain thermostable amboceptor (Dean). This condition is somewhat analogous to that observed in the late stages of diphtheria immunisation, where the animal, though quite resistant to large doses of the toxin, is incapable of producing antitoxin in high concentrations.

Further experiments are necessary, however, and some theoretical points arising from a consideration of the results obtained in this paper will be dealt with in a future communication on this subject.

CONCLUSIONS.

1. The dilution of the fresh unheated serum of the ox and of the rabbit causes a gradual diminution in its opsonising power.
2. The points corresponding to the $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, and $\frac{1}{32}$ dilutions of these normal sera lie on a logarithmic curve, which shows that the phagocytosis is a linear function of the logarithm of the concentration.
3. The intravenous injection of large doses of killed staphylococci entirely alters the form of this curve, the phagocytosis falling quickly to a minimum.
4. The dilution of the serum of an animal immunised against the *Staphyloccus pyogenes aureus* is not accompanied by any marked fall in the opsonising power even at the thirty-second dilution of the serum.
5. That the opsonic index of the undiluted serum is no guide to the manner in which a serum will behave on dilution. It is probable that the amount of immune substance in the serum can be better determined by the dilution method outlined in this paper.

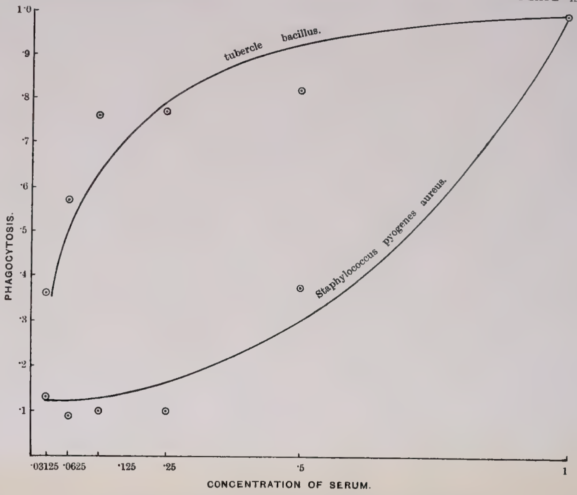

CHART V.—Rabbit Z, showing the phagocytosis in the presence of various dilutions of the serum twenty hours after the intravenous injection with ten agar tubes of killed Staphylococcus pyogenes aureus. Opsonic Index for the tubercle bacillus=0·67, and for the Staphylococcus pyogenes aureus=1·36.

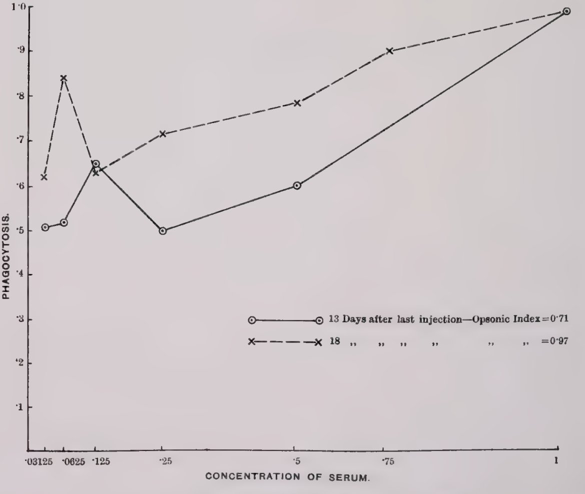

CHART VI.—Immune Rabbit B, showing the phagocytosis in the presence of various dilutions of the serum after numerous subcutaneous injections of killed Staphylococcus pyogenes aureus. (1) Thirteen days after the last injection, and (2) eighteen days after the last injection.

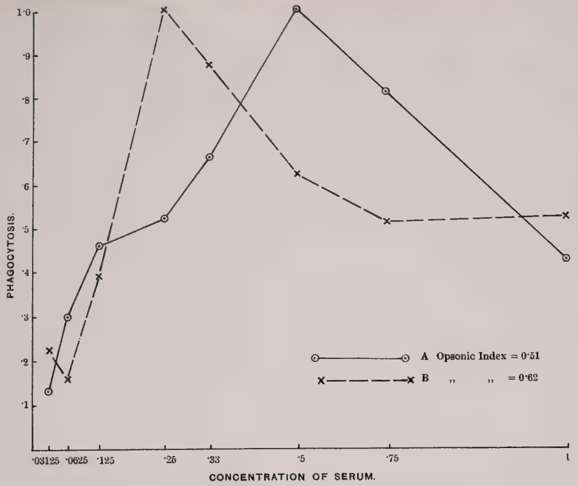

CHART VII.—Immune Horse "Rich," showing the phagocytosis in the presence of various dilutions of the serum of horse immunised by numerous injections of Staphylococcus pyogenes aureus during a period extending over two years. Bled on November 30, 1907 (A), and December 7, 1907 (B).

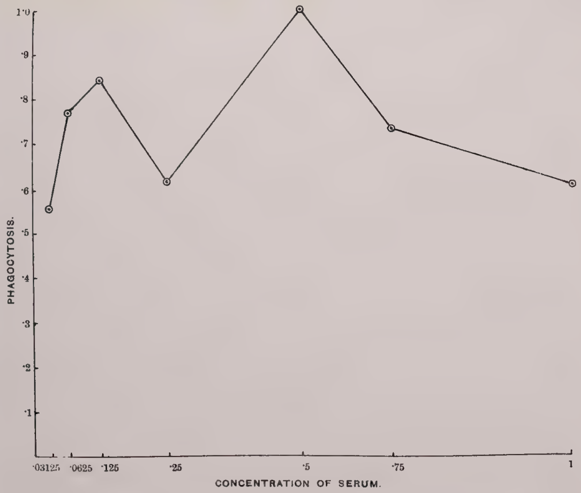

CHART VIII.—Immune Rabbit B, showing the phagocytosis in the presence of various dilutions of the serum after being heated to 60° C. for twenty-five minutes.

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DESCRIPTION OF PLATES XXXVIII.-XLI.

PLATE XXXVIII.

CHART I.—Showing the influence on phagocytosis of various dilutions of the normal serum of the rabbit and of the ox.

CHART II.—Rabbit E, showing the phagocytosis in the presence of various dilutions of the serum twenty-four hours after intravenous injection with three agar tubes of killed *Staphylococcus pyogenes aureus.*

PLATE XXXIX.

CHART III.—Rabbit M, showing the phagocytosis in the presence of various dilutions of the serum after intravenous injection, on May 23, with five agar tubes of killed *Staphylococcus pyogenes aureus,* and again on May 24, with three agar tubes. Bled May 25. Opsonic Index = 0·70.

CHART IV.—Rabbit S, showing the phagocytosis in the presence of various dilutions of the serum one hour, five hours, and twenty-five hours after the intravenous injection with twelve agar tubes of killed *Staphylococcus pyogenes aureus.*

PLATE XL.

CHART V.—Rabbit Z, showing the phagocytosis in the presence of various dilutions of the serum twenty hours after the intravenous injection with ten agar tubes of killed *Staphylococcus pyogenes aureus.* Opsonic Index for the tubercle bacillus = 0·67, and for the *Staphylococcus pyogenes aureus* = 1·36.

CHART VI.—Immune Rabbit B, showing the phagocytosis in the presence of various dilutions of the serum after numerous subcutaneous injections of killed *Staphylococcus pyogenes aureus* (1) thirteen days after the last injection, and (2) eighteen days after the last injection.

PLATE XLI.

CHART VII.—Immune Horse (Rich), showing the phagocytosis in the presence of various dilutions of the serum of horse immunised by numerous injections of *Staphylococcus pyogenes aureus* during a period extending over two years. Bled on November 30, 1907 (A), and December 7, 1907 (B).

CHART VIII.—Immune Rabbit B, showing the phagocytosis in the presence of various dilutions of the serum after being heated to 60° C. for twenty-five minutes.

SNAKE-POISON
AND
SNAKE-BITE.

By
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AND
MAJOR GEORGE LAMB, M.D., I.M.S.

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SNAKE-POISON AND SNAKE-BITE.

By Charles J. Martin, M.B., D.Sc., F.R.S., and

Major George Lamb, M.D., I.M.S.

Venomous snakes are found almost all over the temperate and tropical regions of the world, with the exception of New Zealand and the Oceanic Islands. They are divided by naturalists into two main classes—(i.) poisonous colubrine snakes; (ii.) viperine snakes. These two classes present differences in structure, and also in the properties and toxic action of their poisons.

Distribution of Poisonous Snakes.—The following is a list of the better known and more dangerous poisonous snakes arranged according to their geographical distribution.*

Europe.—Small vipers or adders (*Vipera berus*, *V. aspis*, *V. ammodytes*) are found on the continent of Europe, and *V. berus* occurs in England. These small reptiles, which are very plentiful in some parts of Europe (*e.g.*, Haute Saône in France), are on account of their small size seldom dangerous to man; but the bite of one is followed by severe symptoms, and according to Bottinger, who collected 610 cases of viper-bite, 10 per cent. were fatal. Most of the fatal cases were probably in children.

India, China, and Southern Asia.—In Asia are found the most deadly of the poisonous colubrine snakes, the cobra, *Naja tripudians*, and the hamadryas or king cobra, *Naja bungarus*, and the kraits, *Bungarus cœruleus* and *B. fasciatus*. There are also several species of viper, among which the daboia, *V. russellii*, and the phoorsa, *Echis carinata*, are the most deadly.

America.—The important poisonous snakes of the American continent all belong to the Viperidæ, and are representatives of the sub-family Crotalinæ. In North America are various species of rattlesnake, *Crotalus terrificus*, *C. scutulatus*, *C. durissus* and *C. horridus*, and the copper-head *Ancistrodon contortrix*, and the moccasin, *A. piscivorus*. In South America a number of different species of the genus Lachesis (*L. mutus*, *L. lanceolatus*, *L. atrox*) are found, many of which reach a size of four or five feet or even more. Of these the Fer de lance, *L. lanceolatus*, is perhaps the best known. *Crotalus terrificus* also occurs in Brazil and Northern Argentina.

Africa.—In the North of Africa a species of cobra, *Naja haja*, is common; and two vipers, *Cerastes cornutus* and *Echis carinata*, are responsible for a number of deaths.

In the South are found another species of cobra, *Naja flava*, and two vipers, *Causus rhombeatus* and *Bitis arietans* the puff-adder.

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* The nomenclature adopted in this article is that given by Mr. Boulenger in his *Catalogue of Snakes*, British Museum of Natural History, vol. iii., 1896, to whom we are indebted for assistance with regard to the distribution of snakes.

In *Australia* and Tasmania are a large number of species of venomous snakes, all of which belong to the Colubridæ, although the death-adder, *Acanthophis antarcticus*, simulates a viper by its appearance. The principal species dangerous to man are—*Notechis scutatus*, the tiger snake; *Denisonia superba*, the copperhead; *Acanthophis antarcticus*, the death adder; *Diemenia textilis*, the brown snake, and *Hotechis pseudechis*, the black snake.

Sea-Snakes.—The Hydrophinæ are all poisonous. With the exception of *Platurus* they live entirely in the water, their tails being flattened like that of an eel. They are common in the tropical and subtropical seas of the Indian and Pacific Oceans.

Poison Apparatus and Mechanism of Bite.—The poison is secreted by the cells of a racemose gland, possessing large alveoli which serve as a receptacle for the venom. The glands, one on each side of the head, are placed behind the orbit. In an adult cobra they are about the size of a large almond. The gland is the homologue of the parotid salivary gland in other vertebrates. They are subcutaneous, with the exception of the superior portion of their external surface. Each is enveloped in a dense capsule of fibrous tissue, from which two strong fascial processes extend anteriorly and posteriorly, so as to fix it securely both in front and behind. The gland is in intimate relation with the masseter muscle, which consists of two portions; the superior portion arises from the external surface of the post-orbital bone, the ridge of the parietal, and the crest of the exoccipital, and is inserted into the external surface of the gland-capsule; the inferior portion arises as a flat tendon from the upper part of the posterior half of the internal surface of the gland, and passes downwards deep to the gland, to be inserted into the mandible and dentary. When the snake bites, the gland is, owing to the peculiar insertion of the masseter fibres, powerfully wrung, and the poison expressed into the duct, much in the same way as one expresses moisture from the pores of a cloth by twisting it. That the muscle does indeed act in this way can be seen in the recently-severed head by stimulating its nerve with a faradic current. A large lymph-space surrounds the gland and so permits of this twisting. From the anterior margin of the gland the duct passes forward along the side of the upper jaw. Just in front of the fang it doubles on itself so as to open by a small papilla on the anterior wall of the sheath of mucous membrane which embraces the base of the tooth. The gland-duct is composed of fibrous tissue, and is lined with epithelium. Numerous small gland-alveoli open along its course. Weir Mitchell found a muscular sphincter in the course of the duct in crotalus; but in other species no muscular tissue has been observed.

The fang, except in some of the sea-snakes, is a functional tube with the proximal opening on the anterior surface near the base and the distal opening on the same surface within a short distance of the point. In the centre is a pulp-cavity containing vessels and nerves. During the development of the poison-fang it first becomes flattened antero-posteriorly; ridges then arise on the anterior surface, which, by a subsequent folding of the whole tooth, are brought into contact and coalesce. Thus there are now two cavities in the fang, a round poison canal, and a horse-shoe-shaped pulp cavity. This folding over is incomplete near the base and point of the tooth. In ordinary circumstances the fangs project downwards and more or less backwards, and are almost completely covered up by a sheath of mucous membrane (*vagina dentis*). When the animal is about to strike they become erect. Different snakes vary very greatly as regards the angle through which they can move the maxillary bone and with it the poison-fang. Snakes in which very long fangs are found (Viperidæ) can do this to a very considerable extent; in the colubrines this movement is relatively insignificant.

The upper jaw is formed of the pterygoid behind, and in front by two bars which diverge at a slight angle. The internal of these bars is the palatine, the external the transverse with the maxilla in front, to which last the poison-fang is attached.

The maxilla is fixed anteriorly to the convex inferior surface of the præ-frontal by a joint which permits of a certain amount of gliding, and the posterior surface of the pterygoid is connected by a ligament to the quadrato-mandibular joint. The whole bar is freely movable in an antero-posterior direction. When the pterygo-transverse bar is moved forwards, the maxilla is pushed forward. The forward movement of the maxilla is, however, soon stopped by a strong ligament attaching it to the præ-frontal. If now the pterygoid and transverse advance further, bending occurs at the joint between the transverse and the maxilla, the latter rotates with its lacrimal attachment as a fixed point, and the fang presents in a still more forward direction. The pulling forward of the pterygoid bar is in all cases performed by the spheno-pterygoid and parieto-pterygoid muscles.

Except when the snake is prepared to strike, the poison-channel from the gland-duct to the fang is not completed. As already mentioned, the duct opens on a papilla, which is situated on the anterior surface of the sheath of mucous membrane which embraces the fang (*vagina dentis*). To complete the channel it is necessary that this papilla be brought into apposition with the opening of the poison-canal at the base of the fang. This apposition of the orifice of the poison-duct with the hole in the fang is brought about with wonderful exactness, and in all our experience we can only recollect two instances in which the connexion at this point was leaky. It is accomplished in Australian snakes partly by the pulling forward of the anterior surface of the fang against the opening of the duct, in the erection of the former as described above, and partly by pulling the mucous sheath backwards tightly against the tooth. This latter is accomplished precisely at the right moment, for one of the most important muscles in closing the jaws upon any object bitten, the internal pterygoid, sends off two small tendinous processes which are inserted into this mucous sheath on each side of the tooth, so that the harder the reptile bites the tighter the papilla is pulled into the proximal aperture of the fang canal. The aperture is by this means actually corked. When the snake strikes it closes its jaws like a dog on the part bitten. Thus the poison is discharged at the moment the fangs penetrate the skin.

The Effects of various Reagents on the Toxic Properties of Venoms.—Reagents which precipitate proteids in an insoluble form, or destroy them—such as platinum or gold chloride, silver nitrate, nitric acid in excess, or permanganate of potash—render venoms inert. Solutions of the hypochlorites have the same effect. Carbolic acid if allowed to remain in contact with dilute venom for twenty-four hours destroys its virulence. Prolonged action (forty-eight hours) of caustic potash or soda also destroys venoms. Gastric digestion does not affect cobra venom nor pseudechis venom. Weir Mitchell states that gastric digestion destroys the power of crotalus venom. Fontana found the poison of the European viper to be unaffected by this means. All venoms are destroyed by pancreatic digestion.

Under a variety of conditions the toxins of venoms undergo changes whereby their toxicity is diminished or lost, whereas the power of combining with the corresponding antitoxin is maintained (toxoid formation). This was first pointed out by Drs. Myers and Stephens. Flexner and Noguchi have made some most interesting experiments on the formation of toxoids from the natural venoms. Working with the venoms of the cobra and of the rattlesnake, they found that the neurotoxin was converted into toxoid by keeping a sterile solution of the poison for some time at room temperature ; this change took place more rapidly when the solution was kept at 37° C. The hæmolysin also undergoes rapid toxoid formation when the venom solution is kept at 37° C. Such treatment has, however, no effect on the hæmorrhagin, the constituent of crotalus venom which is responsible for the severe local reaction and for the hæmorrhages, and which these observers consider to be of the nature of a cytolysin for the endothelium of the capillary and arteriole walls. If, however, crotalus poison be treated with hydrochloric acid in weak solution, namely 0·2 to 1 per cent., there is a marked diminution of activity of the hæmorrhagin constituent ; this diminution is of the nature of a toxoid formation, as by injecting rabbits with a venom solution heated in this way a fairly strong anti-hæmorrhagin serum could be prepared. Similarly by treating crotalus venom with a solution of iodine trichloride (0·2 per cent.) toxoid formation of the hæmorrhagin was obtained. Cobra and daboia poisons treated in the same way with iodine trichloride failed entirely to yield any toxoids, the poisons being completely destroyed.

Separation of the Poisonous Constituents of a Venom.—The different toxins, a mixture of which constitutes a particular venom, have in some cases been separated from one another. This has been accomplished in several ways.

(1) Heating to 70° to 100° C., according to the particular venom. Fibrin-ferments, hæmorrhagins, and some neurotoxins, are destroyed at 75°-80°, whereas other neurotoxins and some hæmolysins, *e.g.*, cobra, withstand boiling.

(2) Dialysis or filtering through a gelatin filter supported in the pores of a Pasteur-Chamberland candle, *e.g.*, fibrin-ferments, and hæmorrhagins do not dialyse.

(3) The hæmolysins may be separated from neurotoxins and the other cytolysins by digestion with red blood-cells in the presence of serum previously heated to 56° C.; and the cytolysins for any given organic cell of an animal may be abstracted from a venom by digesting it at 0° C. with an emulsion of such cells. In the same way it has been found that the neurotoxin is absorbed by brain-cells (Flexner and Noguchi).

(4) The hæmolysin of cobra poison can be separated from the neurotoxin by shaking a solution of venom with lecithin dissolved in chloroform. The hæmolysin combines with the lecithin to form an active lecithide, which dissolves in the chloroform and can be subsequently precipitated by ether; the neurotoxin remains in the watery solution. The cobra lecithide is strongly hæmolytic for the red cells of all species with which it has been tried. Its action is very rapid; in concentrated solution almost instantaneous. It is not damaged by heating at 100° C. for six hours, and is much less influenced by a cobra venom anti-serum than the hæmolysin of the original poison.

The watery portion separated out by centrifuging contains the neurotoxic constituent of the venom quite free from hæmolysin. Jacoby has immunised a rabbit with this neurotoxin and has obtained a serum which has a weak neutralising action on the neurotoxin of the original venom. Working in the same way with several other venoms, Kyes has been able to prepare hæmolytic lecithides; that is to say, compounds of lecithin with the hæmolytic constituent of the venom.

The Toxic Value of Venoms.—Different venoms vary greatly as regards their degree of toxicity: also the minimum lethal dose of any given poison varies somewhat for different animals weight for weight.

Sir T. R. Fraser and Major Elliot found that if the minimal lethal dose of cobra venom per kilogramme of body-weight for rats be taken as unity, that for rabbits = 1·2 and that for cats = 20, whereas the minimal lethal dose of the venom of a sea-snake, *Enhydrina valakadien*, the action of which is very similar to that of cobra poison, shewed a totally different relationship for the same three animals. In this case the lethal dose was less per 100 grammes for rabbits than for rats, and if the minimum for rabbits were expressed as units that for rats was 1·5 and for cats 3·3. Sir T. R. Fraser and Major Elliot regard these results as indicating a material difference in the constitution of the two venoms, notwithstanding the similarity of their physiological action. The following table shows the minimum lethal dose of some of the more important poisons for the rabbit expressed in milligrammes per kilogramme of weight. In making such comparisons it is important that only venoms derived from recently captured snakes be employed; for in confinement, notwithstanding forced feeding, their poison soon shews a falling off not only in total amount and in concentration but also in the toxicity of the dried residue. On this account the figures in the table below must be regarded as approximate only.

Species of Snake.	By Subcutaneous Injection.	By Intravenous Injection.
India—Naja tripudians	0·35 milligrammes	...
Naja bungarus	0·35 ,,	...
Bungarus cœruleus	0·08 ,,	0·04 milligrammes
Bungarus fasciatus	2·5–3 ,,	0·7 ,,
Enhydrina valakadien	0·05 ,,	...
Australia—Notechis scutatus	0·05 ,,	...
Diemenia textilis	0·2 ,,	...
Acanthophis antarcticus	0·2 ,,	...
Notechis pseudechis	0·6 ,,	...
India—Daboia russellii	1–2 ,,	0·1 milligrammes
Echis carinata	1–2 ,,	0·05 ,,
Lachesis gramineus	...	2 ,,
America—Crotalus durissus	...	0·25 ,,

The most toxic venoms are evidently those of the Australian tiger snake and the sea-snake, *Enhydrina valakadien*, then comes that of the krait, *B. cœruleus*. The least toxic are those of the banded krait and the green pit-viper.

Description of Venoms.—To obtain venom in a pure state for purposes of chemical examination and experimental inquiry, the best method is to allow the snake to bite an ordinary large watch-glass which has been covered with thin rubber sheeting, such as dentists use. The fangs penetrate the rubber and all contamination by the secretions of the mouth is effectually prevented. Collected in this way the amount of dried venom which a recently captured adult cobra yields varies from ·600 to 1·100 c.c. containing 200 to 370 milligrammes of solid matter; a large daboia yields about the same quantity of liquid venom, namely, from ·6 to 1 c.c., but containing less solid matter—150 to 250 milligrammes; the quantity of poison obtained from the smaller Indian snakes, such as the krait and the phoorsa, is very much less. Calmette obtained the following yields of poison from snakes in captivity:—

Variety of Snake.	Weight in Milligrammes, as discharged.	Weight in Milligrammes, dried.
Lachesis (medium size)	320	127
Crotalus (large)	370	105
Cerastes	123	27

One of us (C. J. M.) found the maximum yield of two species of Australian snakes to be:—

	Weight in Milligrammes, as discharged.	Weight in Milligrammes, dried.
Australian tiger snake (*Notechis scutatus*)	205	73
Australian black snake (*Notechis pseudechis*)	160	94

If a snake be kept for some time in captivity and the poison extracted regularly, the amount of venom that can be obtained by this method is considerably diminished, so that after some month's incarceration perhaps only thirty or forty milligrammes can be got from a full-sized cobra.

Snake-poison is in most instances a clear limpid fluid of a pale straw or yellow colour. Some venoms, however, such as that of *Echis carinata*, have always a certain amount of suspended matter. The colour varies with the degree of pigmentation of the snake, and also with the concentration of the venom. The reaction of the venom is almost invariably acid. The alkalinity observed in some cases may be accounted for by admixture with alkaline saliva. The specific gravity of venoms varies considerably; the average of the poison obtained from 250 cobras was 1110, while the average of the venom from 53 daboias was found to be 1077 (Lamb). The amount of solids contained in these two poisons was found to correspond with the specific gravity; thus, the percentage of solids in cobra venom was on an average 31·5 and in daboia venom 24·4. The variations, however, are considerable, in the case of cobra venom being from 23 to 38 per cent. Most venoms are tasteless, but cobra poison has a disagreeable, intensely bitter taste.

Venoms dry rapidly at 16° to 20° C. in a dessicator over calcium chloride. As they dry they crack in the same manner as albumin or gum-arabic in the same circumstances, and form translucent scales which have suggested a crystalline structure. Dried venoms dissolve again readily and completely in water. Perfectly dry venom in a well-corked bottle keeps indefinitely. Weir Mitchell kept some crotalus venom in his possession twenty-two years without apparent diminution of toxic power. We have had some venoms for thirteen years which are as deadly at the present time as when first procured. Venoms in solution in water do not keep; and the numerous assertions that dried venom deteriorates with age may be accounted for by imperfect dessication. Solutions of venom in glycerin keep indefinitely (Weir Mitchell).

Microscopical examination of pure venom reveals nothing except an occasional epithelial cell; but if the poison be contaminated with fluids from the mouth it contains epithelial scales and salivary corpuscules in abundance, and also bacteria of various kinds.

Composition of Venoms.—It has long been known that snake venoms contain albuminous bodies in solution; but, owing to ignorance of the toxic properties of proteids, the active principle was sought for in other constituents. Weir Mitchell was the first to demonstrate that the poisonous properties of rattlesnake venom reside in the albuminous constituents; and the work of this accomplished author on the venom of the rattlesnakes formed the first step in our knowledge of toxic proteids. Since Weir Mitchell's work was published his results have been extended by other observers to a number of different kinds of snakes.

Venoms consist for the most part of solutions of modified proteids, and all attempts to separate the toxic principles from such proteids, a separation which has, for example, been accomplished by Jacoby and Hausmann for ricin and abrin, have hitherto been unsuccessful. Accordingly, at the present time we must regard such toxic principles as residing in some special grouping of a portion of the atoms in the complex venom proteid molecule; although it is possible that this is a side-chain and may be ultimately capable of differentiation as a separate entity. Venoms are by no means simple solutions of one poisonous substance; the more they have been studied the more complex have they been shewn to be. The analysis of their physiological actions has proved them to be made up of a great many more constituents than would be imagined from their chemical examination. Different venoms have been found to contain one or more of the following:—(1) A powerful fibrin-ferment; (2) an anti-fibrin ferment; (3) a proteolytic ferment; (4) various cytolysins—capable of acting upon red blood-cells, leucocytes, endothelial cells of vessels, nerve-cells, and the cells of various other tissues. These cytolysins are probably distinct for each variety of cell and are of the nature of amboceptors; (5) agglutinin for red blood-cells, etc.; (6) an antibactericidal body, of the nature of anti-complements; (7) a neurotoxin or neurotoxins with affinities for all nerve-cells and especially for the cells of the respiratory centre in the bulb, although the special affinities of the neurotoxic constituents vary somewhat with each venom; (8) a neurotoxin with an affinity for nerve-endings in muscle, and for those in the diaphragm in particular; (9) a substance which causes greatly increased tone in cardiac muscle, and if in sufficient concentration systolic standstill of the isolated heart. This substance produces also a similar stimulating action on the muscle of the arterial walls. No venom yet studied is possessed of all the above constituents, and the dissimilarity in the effects produced upon man and animals after poisoning with the venoms of different snakes is due to the proportions in which the different groups of toxic substances occur in the particular venom. While in different venoms we may recognise substances with the same physiological action, it is not to be taken for granted that such substances are identical. Recent experiments with the sera of animals immunised with different venoms have shewn that many of these constituents from different venoms, even when incapable of differentiation by their physiological action, display their non-identity by the inability of an anti-serum prepared with one poison to neutralise the constituents of similar action of another poison; or expressed in terms of Ehrlich's nomenclature, that, although the toxophorous groups may be similar, the haptophors are dissimilar. This has been especially shewn in the case of the hæmolysins of venoms.

Further, by means of the serum precipitin test it has been shewn that the proteids of one venom may differ from those of another poison, although the physiological actions of the two venoms are apparently identical. Thus, the serum of a rabbit immunised with pure cobra venom causes a copious precipitum with cobra venom but none whatever when mixed with the venom of the king cobra, the physiological action of which poison cannot be differentiated experimentally from that of the cobra. The importance of these facts on the specificity of venoms in connexion with the serum therapeutics of snake-bite will be at once evident, and we shall have to return to this question later when discussing the treatment of cases of snake-venom intoxication.

Behaviour of Venoms with Reagents.—Venoms give all the reactions characteristic of proteids with the usual proteid-reagents. On dialysis more or less of the proteid in venom is thrown out of solution, the amount varying with different venoms. To judge by the hitherto recorded observations it is greatest with crotalus poison, and least with that of the cobra. Saturation with ammonium sulphate completely precipitates all the proteids, and the filtrate is innocuous. Saturation with sulphate of magnesium, or chloride of sodium, and partial saturation with ammonium sulphate produce a separation of the proteids in venoms; and in the case of most venoms both the precipitate and the filtrate are possessed of poisonous properties. Absolute alcohol *in excess* entirely precipitates all proteids from venom solutions, and the filtrate is not poisonous. After sojourn under alcohol the bulk, and in some cases the whole, of the proteid is readily soluble in dilute saline solutions, and the solution possesses the same properties and nearly the same toxicity as the original venom.

Effect of Heat upon Venoms.—Solutions of all snake-poisons so far examined contain a considerable amount of proteid coagulable by heat, which, according to the observations recorded below, appears not to vary greatly in the poisons of different species of snakes. The following table gives the percentage for the venoms of three Indian species—two colubrine and one viperine—and of crotalus:—

	Cobra.	Banded Krait.	Daboia.	Crotalus.
Coagulable proteids . .	24	20	25	25
Non-coagulable proteids .	76	80	75	75
Temperature at which coagulation takes place .	70°-80° C.		70°-80° C.	60°-70° C.

Although the amount of proteid coagulated by heating varies so little, the effect produced upon the toxicity of the different venoms by bringing their solutions to a temperature of 70-80° C. for a few minutes is strikingly different. Cobra poison, for instance, is little if at all reduced in toxicity by such treatment; whereas the venoms of the viperine snakes, crotalus and daboia, are rendered nearly but not quite inert. The effect upon a number of other venoms, such as those of the Australian snakes, *Notechis scutatus* and *N. pseudechis*, is intermediate; they lose some portion of their toxic qualities whilst retaining others. When a particular coagulable constituent is toxic, raising the solution to the temperature at which this coagulates renders it inert. If the heating be carried to the boiling-point a further gradual diminution in toxicity is brought about in all venoms, and continuous boiling for a few hours deprives them of all poisonous action.

The effect of heat is two-fold:—(1) a more or less rapid destruction of a poisonous constituent of some venoms by coagulation; (2) a gradual deterioration by prolonged heating, the effect of which is more rapid the higher the temperature and the weaker the solution.

Perfectly dry venoms may be submitted to a temperature slightly above 100° C. without diminishing their toxic power. Some of the proteids present in venoms are classified with difficulty in our present system. A classification which is determined (amongst other reactions) by solubility in water or dilute saline solutions, coagulation by heat, and solubility or insolubility in water or saline solutions after prolonged sojourn under alcohol, is a very arbitrary one ; and it is to be expected that, as our knowledge of proteids increases, more individuals of the group will be found which refuse to fall into line with the members hitherto known. The toxic proteids of the poison of pseudechis are completely precipitated by absolute alcohol in excess ; yet after six months under alcohol these proteids dissolve readily and entirely in dilute saline solutions. This poison, nevertheless, contains a body which, by its solubility in water and coagulation by heat, would lead one to class it with albumins. The solution of the alcoholic precipitate also coagulates on heating. Venoms contain proteids which possess on the one hand characteristics of the albumins or globulins, and on the other those of proteoses. This has led to a certain amount of confusion—one observer classifying a venom proteid with the albumins or globulins because of its behaviour when heated in solution, another preferring to place the same proteid amongst the albumoses on account of its continued solubility in water or dilute saline solutions after prolonged sojourn under alcohol.

Analysis of the Physiological Action of Snake Venoms.—*Method of Absorption of Venom.*—Venoms may enter the body by a number of channels. The onset and train of symptoms vary with the rapidity with which they reach the circulation. In cases of snake-bite the poison is usually deposited in the subcutaneous tissue, whence it reaches the general circulation, principally by absorption through the blood-vessels. When introduced into a serous cavity, absorption is much quicker ; and, if introduced directly into a vein, the effects are manifested instantaneously. Venoms are readily absorbed from the conjunctiva. Feeding an animal with snake poisons, even when the diet contains daily a hundred times the fatal dose, does not produce any symptoms of poisoning, provided there be no abrasion of the mucous membranes. The poison has not been recovered from the fæces, so that it must be destroyed by the digestive juices. As mentioned above, pancreatic juice has been found to destroy all those venoms with which the experiment has been made. Major Elliot has shewn that when the actions of the various digestive juices do not come into play cobra-poison can be absorbed from the small intestine. There is a striking difference in the effects produced by subcutaneous and intravenous injection of moderate quantities of those venoms which contain fibrin-ferment (see p. 20). Even very small quantities of such poisons intravenously introduced occasion extensive intravascular clotting and sudden death ; whereas, placed under the skin, unless in relatively large quantities, the thrombosis is only local, and this in itself retards the further absorption of the poison.

General.—During recent years not only has a considerable amount of experimental work on the physiological action of different snake venoms and on the individual poisons contained in them been carried out, but great strides have been made in our knowledge of the mode of action of toxins, cytolysins, and enzymes, to which venoms are closely analogous. The poisonous constituents of venoms, as has been previously mentioned, are modified proteids. They belong to the first of the two main groups into which Ehrlich has divided substances possessing pharmacological action. Ehrlich's view of the action of this group is bound up with his conception of the nature of the living protoplasmic molecule as a nucleus with side-chains of various descriptions. According to this hypothesis, assimilation of food-stuffs consists in the linking on to the central molecule of the food-molecule as a fresh side-chain. The common features of substances of this first class are their close resemblance to assimilable substances or food-stuffs, and the special affinity exhibited by different bodies of this class for particular cells. They are thus, so to speak, selectively absorbed by one class of cells from the circulating fluids, so that they gradually become attached to them, and to them only. In this way even excessively small quantities exercise a potent influence.

This first group of Ehrlich includes all those bodies with chemical character more or less allied to proteids, to which the toxins and enzymes belong. All of these, whether produced by the activity of bacteria—as tetanus or diphtheria toxins,—or by higher plants—as ricin, abrin, or the toxin of grass-pollen, which is responsible for hay-fever,—or in animals—as rennet, trypsin, or the venoms of snakes,—are produced by living cells. These toxins and enzymes possess the capacity of attaching themselves to particular cells or molecules, much as a key fits a particular lock or series of locks; and being affixed to the cell they occasion such a disturbance of the normal activities of the cell that its function is disturbed or destroyed, and in some cases the cell itself is disorganised. They also possess in common the capacity of arousing the formation of anti-bodies in excess when injected into an animal at suitably spaced intervals.

For these and other reasons snake venoms must be classed with toxins, lysins, and enzymes, and a general survey of the principal actions of their constituents shews that they may be grouped in three classes. (1) Neurotoxins, which combine with nerve-cells and render them inactive. These often exhibit a strikingly selective action upon particular groups of cells. (2) Cytolysins, for a number of animal cells, such as red blood-corpuscles, endothelial cells of blood-vessels, leucocytes. (3) Fibrin-ferments, the injection of which occasions intravascular clotting. This does not exhaust the whole of the poisonous components of some venoms which contain, in addition to representatives of one or more of the above groups, substances possessed of actions, such as that of cobra-poison on the muscular tissue of the heart and arterial wall, or that of a constituent of rattlesnake-poison which inhibits the normal bacteriolytic action of the serum. There are also proteolytic ferments in crotalus and some other venoms, but these are of subsidiary importance, and along with others will be mentioned later.

Poisons which possess a special affinity for nerve-cells are present in greater or less amount in all venoms, but, speaking generally, they are of greater potency, and are present in greater amount in the venoms of colubrine snakes than in those of the Viperidæ.

One or more cytolysins exist in most venoms, and have been shewn by Flexner and Noguchi to be of the nature of amboceptors; after these have combined with the cells the latter undergo destruction and solution under the influence of complements contained in the animal's own serum. Many venoms exert a destructive action upon more than one class of cell, an action brought about by the presence of a plurality of cytolysins (Flexner and Noguchi). The action of some of the best known cytolysins in venoms will be discussed in dealing with that of snake-poisons upon blood and blood-vessels.

The fibrin-ferments exist in greater or less amount in the venoms of viperine and in many of the colubrine snakes. Their action is important in bringing about the rapid death of small animals on which these reptiles prey; but this result seldom occurs in cases of snake-bite in the human subject, unless the poison has accidentally been introduced directly into a vein.

Every venom does not contain toxins belonging to all three of the above groups, and the difference in the results of the injection of the poison of different snakes is largely due to the variation in the proportion in which representatives of the above groups exist in venoms. The constituents of different venoms belonging to the same group are not usually identical, but display differences in the details of their action; and even when this action is similar, the absence of complete identity is shewn by the observation that an anti-body produced against one particular toxin is either without effect upon others or else requires a considerably increased amount to neutralise it.

Action of Venoms on the Nervous System.—The determination of the exact physiological action of a poison on the nervous system of the higher animals is always difficult. The difficulties are enormously increased when the poison, as is the case with some snake venoms, contain constituents which profoundly affect the blood and the whole circulatory apparatus also. In such a case special precautions have to be taken to distinguish effects which are due to the primary action of the poison on nervous tissue from those which are secondary to changes produced on the circulation and blood. The early experimenters did not realise that most viperine and many colubrine venoms contain fibrin-ferments, which occasion extensive intra-vascular clotting if introduced directly into the blood stream even in minute quantities, and often do so when subcutaneously injected in larger amounts into small animals; this has naturally been the cause of the greatest confusion in interpreting some of their experimental results, especially those in which the poison was introduced into a vein. The striking manifestations of disturbance of nervous activity which follow sudden cessation of the circulation have, in ignorance of this property of some venoms, been attributed to the direct action of the poison upon the nervous system.

The general feature of poisoning by all venoms consists in depression of nervous activity, with gradual onset of inco-ordination, lethargy with diminished reflex response, coma, and cessation of respiration. The particular feature of the poisoning by individual snakes is determined by the potency and character of the neurotoxin contained in its venom, and the relation of this neurotoxin to constituents toxic for other cells of the organism.

The venoms of colubrine snakes, the physiological action of which has been studied in some detail, are those of the cobra, hamadryas, the kraits, the sea-snake, *Enhydrina valakadien*, the Australian snakes, *Notechis scutatus*, *N. pseudechis*, and *Acanthophis antarcticus*. With all of these venoms the paralytic action upon the nervous system is the best marked feature of their action. Frogs injected with these poisons soon lose all reflex activity, and their spinal cords become totally inexcitable. Mammals become progressively paralysed until death ensues from paralysis of the respiration ; if, as is the case in cobra-poisoning, the circulation be well maintained, the animal may be kept alive by artificial respiration for hours, during which period reflex action is entirely abolished. Cobra-venom is particularly suitable for this experiment, as owing to the direct stimulative action of this venom upon the arterioles the blood-pressure does not fall.

Respiratory paralysis is quickly brought about in mammals by bringing the poisons into contact with the central nervous mechanism which controls respiration ; this can be done by placing a few drops of a solution of venom in the fourth ventricle.

If, after the subcutaneous injection of one of the above venoms, simultaneous records of the respiration and blood-pressure be taken during the poisoning of a dog or rabbit, the circulation is found to be fairly well maintained up to the time that all respiratory movements have ceased, and may subsequently undergo a marked asphyxial rise, shewing that the vasomotor centre is still functionally active. If, however, asphyxia be obviated by artificial respiration, the vasomotor centre fails shortly after the respiratory centre, and the pressure slowly falls to a few millimetres of mercury pressure, except in experiments with cobra-poison and that of *Bungarus cœruleus*, where direct arterial constriction occurs.

From what has just been said it is clear that certain groups of nerve-cells in the medulla exhibit a special susceptibility to the action of the neurotoxins in these venoms, and that the cells connected with the central nervous mechanism of respiration are particularly affected. The neighbouring cells in the bulb participate early in the paralysis, so that more than one observer has drawn attention to the similarity of the symptoms of snake poisoning to those of the late stages of bulbar paralysis.

The influence of the neurotoxins of venoms upon nerve-cells is not confined to an interference with function ; in cases where they have been looked for, structural changes in the nerve-cells have been found to follow injections of these poisons. Kilvington found that the cells of the spinal cords of rabbits poisoned with the venom of *Notechis scutatus* exhibited degenerative changes resembling those described by Dr. Mott in abrin poisoning. Preparations stained by Nissl's method shewed chromatolysis and ultimately disappearance of all staining particles. No swelling of the cell occurred ; the nuclear outline vanished, and in some cases disappeared, but the nucleolus remained. Many of the cells were represented by mere "ghosts," which suggested a cytolytic process. Unless the animals survived the poisoning for four or five hours no changes in nerve-cells could be detected.

The nervous systems of monkeys killed with the venom of cobra, of *Bungarus fasciatus*, of *Bungarus cœruleus*, and of daboia, have been examined microscopically by Lamb and Hunter. With the venoms of the first four they produced an acute chromatolysis of the ganglion-cells throughout practically the whole cerebrospinal nervous system. These changes were, as a rule, most marked in the cord, less so in the cerebral cortex, and least apparent in the pons and medulla; the longer the animal lived after receiving the venom the more extreme was the chromatolysis. No changes were visible in the ganglion-cells of monkeys dying within two hours of injection. In none of the animals did the peripheral nerves shew signs of degeneration. No changes in the nerve-cells were observed in monkeys killed with the poison of *Daboia russellii*, although one of them lived for sixty hours. It has already been mentioned that venoms contain crystolysins for red blood-cells, leucocytes, endothelium, etc. In the case of these cytolysins the mechanism has been shewn by Flexner and Noguchi to be analogous to serum-hæmolysis, the venom constituent acting as an amboceptor, and the cell destruction being brought about by a complement present in the animal's own serum. The appearances seen in the nerve-cells of animals which have lived for two or three days after snake poisoning strongly suggest that the action of the neurotoxins may also belong to this category. This view would explain both the latent period which ensues before the onset of paralysis, when only minimal fatal doses are employed, and also why no changes can be detected in the cases in which death ensues in a few hours.

With viperine poisoning direct action upon the nervous system is not nearly so marked as with the venoms of the colubrines. The neurotoxins of viperine venoms are less potent, or present in much smaller amount, while the actions of the other constituents in these poisons are more pronounced.

Of the poisons of the viperine snakes, those of *Crotalus*, *Daboia russellii*, and *Lachesis anamallensis* have been best studied with regard to their action upon the nervous system. Symptoms of general depression of all nervous activity occur under the influence of these poisons, but the respiratory nervous mechanism does not appear to be selectively affected. As will be mentioned later, these poisons occasion a striking fall of blood-pressure, which is due to an action upon the vasomotor mechanism, and is attributed by Major Rogers to paralysis of the central mechanism. Simultaneously with the fall of pressure the respiration becomes shallower and slower until it ceases.

There exist also in some venoms, *e.g.*, those of the cobra (Brunton and Fayrer), king cobra, kraits (Rogers), *Enhydrina valakadien* (Fraser and Elliot), neurotoxins which act upon the nerve-endings in voluntary muscle, or else the neuro-muscular junctions, in a manner analogous to the alkaloid of curara. This action is more easily made evident in experiments in which the poison takes some hours to kill, or in those in which death from central respiratory failure has been obviated by artifical respiration. The phrenic nerve-endings appear to be particularly susceptible to this action, for it is found that the diaphragm ceases to respond to the stimulation of the phrenic nerves at a time when irritation of the brachial or sciatic plexus with the induced current produces good contractions of the corresponding muscles. This curara-like action is not always present in venoms which kill by causing cessation of respiration. The poisons of the Australian snakes, *Notechis scutatus* and *N. pseudechis,* both of which contain powerful neurotoxins with a special affinity for the respiratory centre, have no such action, nor was Feoktistow able to discover any such action with the venoms of *Crotalus* or *V. berus.*

Chronic Intoxication due to Action upon the Nervous System—The poison of the banded krait *Bungarus fasciatus* contains a neurotoxin which possesses an action not observed in poisoning by other snakes. Wall first pointed out that in acute poisoning by this venom the symptoms resembled those of cobra intoxication, and death took place from paralysis of respiration; if, however, a small quantity of poison was injected, the animal shewed no acute symptons, but after a few days, in which no departure from health was noticed, suffered from a chronic wasting illness characterised by loss of appetite, great depression, irregular temperature, and excessive muscular weakness. Purulent discharges took place from the eyes and nose, and albumin was found in the urine. The muscles became more and more atrophied, and the increasing weakness passed into a condition of general paralysis and death. Dr. W. Hunter and one of us (G. L.) examined the nervous systems of monkeys which succumbed in about ten days to this chronic intoxication, and found extensive primary degeneration of most of the nerve-cells in the brain and cord. The majority of the cells were vacuolated with deeply stained plasma, in which were scattered dust-like granules, the remnants of the Nissl-bodies. Many of the cells were reduced to simple outlines without any granulation whatever.

Effect of Venoms upon the Respiration.—The selective activity of the venoms of colubrine snakes upon the respiratory cells in the medulla, which have hitherto been the subject of inquiry, has been already mentioned. It has further been pointed out that some venoms also exert a curara-like action which is particularly manifested upon the nerve-endings of the phrenics in the diaphragm, so that failure of respiration, brought about in one or both ways, is the cause of death in poisoning by the venoms mentioned.

Perhaps one of the most striking demonstrations of this action was the early experiment of Sir Lauder Brunton and Sir J. Fayrer on the fowl. If cobra venom be injected into a fowl, the creature becomes lethargic and inco-ordinate; the head drops on the breast, to be occasionally jerked up again, as happens with a man sleeping in a chair. Presently the respiration, which was at first accelerated, becomes slower and more and more shallow, until it finally ceases; meantime the comb has become livid. If artificial respiration be performed, the comb again recovers its bright red colour, again to become livid on its cessation. This may be repeated a dozen times. The obvious interpretation of this experiment is that in cobra poisoning the respiratory movements are rendered impossible whilst the circulation is well maintained. The same facts are seen when mammals are the subject of experiment; after a preliminary increase in respiratory activity, a progressive diminution in number and amplitude occurs, until respiration ceases altogether. Small animals poisoned with cobra venom have been kept alive for four hours by artificial respiration; and Vincent Richards records a case in which the circulation was maintained, by means of artificial respiration, for thirty hours in a man poisoned by a cobra. This selective action of a poison for the respiratory mechanism is seen in a most marked degree with the poisons of the cobras and the sea-snake, *Enhydrina valakadien*. With the other colubrine venoms the vasomotor mechanism becomes affected shortly after the respiratory, so that the blood-pressure falls and circulatory failure follows respiratory failure within a few minutes or an hour, according to the conditions of the experiment and the venom employed. The respiratory movements are, as mentioned above, rendered impossible by some venoms in two ways: (1) action upon the nerve-cells in the central nervous system; (2) interference with the passage of nervous impulses at the nerve-muscular junctions of the phrenic nerve-endings. Most snake venoms possess only the former action, but those of the *Enhydrina*, cobras, and kraits are endowed with poisons operating in both ways. In the case of those venoms possessed of both actions, the influence upon nerve-cells is the more rapid and important; so that it is only under conditions of slower poisoning that the curara-like action can be demonstrated to its full extent.

The effect upon respiration of the venoms of those viperine snakes, an analysis of the action of which has been undertaken—*Crotalus*, *Ancistrodon* (Weir Mitchell), *Daboia*, puff-adder, and *Lachesis* (Rogers), —is not so specific as in the case of the colubrine venoms. With these viperine poisons the effect upon the nervous system as a whole is less intense, and no individual group of nerve-cells appears to be strikingly picked out. When simultaneous records of respiratory movements and blood-pressure have been taken, the arterial pressure has been found to fall to a dangerous extent, at the same time that the respiratory tracing has shewn diminution in rate and amplitude. The dependence of respiratory activity upon the arterial pressure has been shewn in the case of chloroform poisoning by Drs. L. Hill and Embley, so that it is rather difficult to ascertain exactly how much direct paralysis of respiratory cells co-exists when complicated by the indirect effect of a greatly lowered blood-pressure. What appears to happen is that when the blood-pressure has fallen to a sufficient extent, the respiratory cells, which are now half-paralysed, cease to be active and respiration fails. In no case has absolute paralysis of respiration been observed in association with circulatory efficiency.

Effect of Venoms upon the Circulation.—Venoms may influence the circulation either by their direct action upon the muscle of the heart or arterioles, or indirectly through the nervous system.

Action on the Isolated Heart.—Most venoms, unless they are much more concentrated than ever occurs in cases of poisoning, exert but little influence upon the isolated heart of cold-blooded animals. On the other hand, the efficiency of the circulation is soon impaired in experiments with all venoms, except those of cobra and *Bungarus cœruleus*, but to what extent this may be due to direct action upon the heart itself has not been clearly shewn, for all venoms ultimately paralyse the cells of the nervous vasomotor mechanism.

The poisons of the cobras and the common krait, *Bungarus cœruleus*, and to a much less extent the venoms of *Enhydrina valakadien* and *Notechis scutatus*, the Australian tiger-snake, have, however, an effect upon cardiac muscle which was first observed by Sir Lauder Brunton and Sir J. Fayrer, and has recently been more completely studied by Sir T. R. Fraser and Major Elliot, who found that cobra venom in dilutions of 1 in 10,000,000 and upwards exerts a stimulating action (induces increased tone) upon the isolated hearts of frogs or mammals. As the strength of the poison in solutions with which the heart is fed is increased, the effect becomes more marked until the heart is brought to a standstill in systole.

Direct Action upon the Arterioles.—The venoms of the cobra and common krait exert a stimulating action upon the muscle of the vessel-wall similar to that just described in the case of cardiac muscle. This direct action of these poisons is the reason why the blood-pressure is maintained at or above its normal height during poisoning with these venoms. Major Rogers has shown that constriction of arterioles is also produced by the local action of the venom of Russell's viper.

Effect upon the Blood-Pressure.—Records of the blood-pressure during poisoning by quite a considerable number of snake-venoms have been made. Many of the older results, and especially those in which the poison was injected intravenously, have been erroneously interpreted, as it was not known that many venoms produced intravascular clotting. This source of confusion must be eliminated before the direct effect of a poison upon the heart or vasomotor mechanism can be estimated. More recent workers have been able to obviate this difficulty.

The venoms of cobras exercise a peculiar effect upon the blood-pressure; this is due to their stimulating action upon the heart and blood-vessels, and the simultaneous rapid paralysis of the respiration before the other nervous centres. Consequently the picture presented is a record in which the blood-pressure remains at its normal height, or even slightly raised, until the respiratory movements are no longer adequate to aerate the blood sufficiently. The vasomotor and vagus centres which are not yet paralysed respond to the stimulus, the pressure rises, and the heart slows much as in asphyxia in a partially curarised animal. If artificial respiration is not carried out, the pressure slowly falls, as the heart fails from want of oxygen, to 20-30 mm. of mercury. If artificial respiration is maintained, the pressure keeps up sometimes for hours and only slowly falls. In experiments with the venoms of other colubrine snakes, such as *Enhydrina*, *Bungarus fasciatus*, *B. cœruleus*, *Notechis scutatus*, *Notechis pseudechis*, and *Acanthophis antarctica*, the results are different. In all of these cases the blood-pressure, even if well maintained at first, soon shews signs of failing, *pari passu*, with the paralysis of respiration ; and although in some cases respiration may cease before the onset of marked circulatory depression, the latter soon follows, and artificial respiration is incapable of preventing a slow fall of blood-pressure to zero. Simultaneously with the fall in pressure a rise in the volume of the spleen or bowels has been observed, so that vasomotor paralysis is in large part responsible for the failure of the circulation.

The effect of viperine poisons upon the blood-pressure is to produce a considerable fall, which is to a large extent due to the action of these poisons upon the nervous vasomotor mechanism. The influence of the venoms of *Daboia*, puff-adder, *Lachesis* and *Crotalus* upon the circulation is more striking than the effect upon the respiration, and the great depression which is a symptom of viperine poisoning may no doubt be explained in this way.

The effect of all the venoms which cause fall of blood-pressure is exaggerated when they are introduced intravenously.

Action of Venoms on Blood-Plasma.—Most, if not all, of the poisons of viperine snakes, and the venom of a number of the Australian colubrines also, contain fibrin-ferments. These venoms cause clotting in every kind of plasma in which coagulation has been suspended by artificial means ; and if introduced with sufficient rapidity, as by intravenous injection, into the blood-stream of an animal, whether reptile, amphibian, bird, or mammal, intravascular clotting takes place and consequently cessation of the circulation. This is indeed the way the death of small animals, which form the prey of these snakes, is usually brought about. The snake injects a large quantity of venom, relatively to the minimal lethal dose, and the effect is the same as when smaller quantities are introduced directly into a vein. In snake-poisoning in man, however, these fibrin-ferments do not play an important part, as they do not reach the circulation with sufficient rapidity to produce general thrombosis. The effect upon the plasma in these circumstances is in the opposite direction, and the blood is found to possess diminished coagulability, or even to fail to clot at all. This negative phase only occurs in the living body. It establishes a kind of immunity against the ferment, for when the blood has arrived at this condition the further addition of large quantities of ferment fails to cause clotting. The mechanism of this immunity has not been ascertained.

The poisons of the cobras contain a substance the direct action of which prevents the coagulation of blood. This effect is manifested either in the body by the presence of a non-coagulable blood after injection of cobra-poison or *in vitro* upon oxalate- or citrate-plasma.

Plasma kept fluid by either of these means, to which cobra venom has been added, fails to clot on the subsequent addition of lime. The venoms of the other Indian colubrine snakes exert no influence upon blood-plasma.

Effect of Venoms on Blood-Corpuscles.—The effect of crotalus venom upon red blood-corpuscles was pointed out by Weir Mitchell and Reichert, who found that the red corpuscles lost their biconcave form and became spherical and sticky, so that they adhered together in strings. Subsequently the hæmoglobin dissolved out and they became invisible. Ragotzi observed the same dissolution of corpuscles with cobra poison, and Feoktistow with the poison of *Pelias berus*, the common viper of Europe. The venoms of the Australian colubrines, *Notechis scutatus* and *N. pseudechis*, possess powerful hæmolytic action, and hæmolysins have in recent years been shewn to exist to some extent in the poisons of all the snakes that have been investigated

The activity of these hæmolysins for the corpuscles of different animals varies enormously, and the venoms of some snakes contain hæmolysins more potent for the corpuscles of one species of animal, whereas those of another snake are more active in hæmolysing the blood-corpuscles of a different species. Hæmolysis is seldom, however, a marked feature of human poisoning by any venom, because the dose of a venom necessary to destroy life by other means, *e.g.*, by paralysis of respiration, is very much less than the quantity necessary to produce any very extensive destruction of blood-cells.

The most marked hæmolysis *in vivo* is produced by the venom of the Australian black snake *Notechis pseudechis*, when sub-minimal lethal doses are injected into dogs. In some cases the red cells were reduced in two days to one half their original number, and the urine was saturated with hæmoglobin. This large effect was not seen when other animals were experimented upon.

In recent years light has been thrown upon the mechanism of venom hæmolysis by Flexner and Noguchi, and Kyes and Sachs, who have shewn that the phenomenon is in many respects analogous to serum hæmolysis and bacteriolysis. On this account experiments with venoms have attracted an additional interest, as they have been found suitable for employment in fundamental experiments concerning cytolysis in general. Flexner and Noguchi found that certain washed blood-corpuscles were not hæmolysed by venom, but that when the previously separated serum was added solution took place. They concluded, therefore, that in the process of hæmolysis venoms act only as intermediary bodies or amboceptors, and require a complement to complete the reaction. This interpretation of the action of hæmolysins in snake venom has been confirmed by Kyes and Sachs, who found, however, that the washed cells of some animals, *e.g.*, dog, horse, man, were hæmolysed to some extent by cobra venom alone, and explained this as due to those cells containing complement within themselves. In further experiments they showed that lecithin was capable of acting as complement in the case of cobra venom, so that their explanation of endocomplement is probable. One of us (G.L.) has confirmed and extended these observations on venom hæmolysis, and has also demonstrated that in the absence of complement or complementoid the hæmolytic amboceptor of venom is not taken up by the red cell.

Action of Venoms upon white Blood-cells.—The effect of most venoms upon leucocytes has not been studied. The effect of 1 per cent. solution of the venoms of two Australian snakes upon the white cells of the frog and mammals upon a warm stage shewed that after a few minutes all movements ceased, the cells became granular and the nuclei more distinct, and in half an hour to one hour many of them were dissolved. Besides inhibiting the movements of leucocytes outside the body, these snake venoms also interfere with their vital activity in the body, as shewn by the following experiment, which is one of a series with similar results. Two small pieces of sterilised ·sponge, about 1 mm. cube, were aseptically introduced into the abdominal wall of a guinea-pig. One of these little sponges had been soaked in 7 per cent. solution of NaCl containing 1 per cent. of venom, the other in the saline solution without the venom. Both sponges were pushed about a centimetre away from the incision, which was afterwards drawn together by a horse-hair suture and covered with collodion. After two hours œdema occurred around the venom-containing sponge, but not around the other. At the expiration of five hours the animal was killed, and both sponges were carefully withdrawn and plunged into absolute alcohol. Sections of the two sponges treated in the same way presented very different appearances. The control was infiltrated with leucocytes which stained well with ordinary nuclear stains; the other contained leucocytes near the margins only, and many of these were broken down and took the stain badly or not at all. From these sponge experiments it was concluded that whereas into the control sponge the leucocytes by their amœboid movements could penetrate unharmed, in the other sponge their activity was paralysed; they succumbed, and were eventually disintegrated by the solution of the venom.

Flexner and Noguchi have since made a more complete and accurate study of venom leucolysis. They used the poisons of cobra, rattlesnake, and moccasin, and found ·002 per cent. solutions effective *in vitro*. After a time, depending upon the concentration of the venoms, motility ceased, followed by granulation and disintegration. They found that the leucolysin was distinct from the hæmolysins in these venoms, and that the process was of the same nature as hæmolysis, and required the addition of a complement, which was contained in the animal's own serum.

Effect on the Blood-vessels.—The occurrence of hæmorrhages both locally and all over the body is most marked after poisoning by the venoms of South American vipers of the species *Lachesis*. It is particularly characteristic of the action of the crotalinæ, and it is a prominent feature of all viperine poisoning. It also occurs, but to a small extent, with some of the Australian colubrines. Weir Mitchell first investigated the mechanism of the phenomena of hæmorrhagic extravasation. He prepared the mesentery of a warm-blooded animal for microscopical observation on the warm stage, and then applied crotalus venom to it. He noticed an almost immediate dissolution of the continuity of the capillary walls with which the poison came in contact. This occurred suddenly, and was not preceded by any diapedesis of white corpuscules. The blood escaped, without previous indication, from those capillaries which were nearest to an arterial twig, and in which, therefore, the hydrostatic pressure was highest. Ligature of the vessels to the part prevented further hæmorrhage.

Flexner has given the name of *hæmorrhagins* to the constituents of venoms, which destroy the continuity of vessel-walls, and looks upon them as cytolysins for endothelial cells of blood vessels. Careful histological observations of the effect of hæmorrhagins shew that there is a definite solution of continuity of the endothelial lining of the capillaries, so that this view is no doubt correct.

Examination of the Gases in Blood after Poisoning with Venom.—Beyond setting it free from the corpuscles, snake venoms exert no influence on the hæmoglobin of the blood: it is still capable of taking up oxygen and parting with it as under normal conditions. In an animal poisoned with the venom of the Australian black snake the quantity and also the tension of oxygen in arterial blood, except immediately preceding death when the respiration and circulation were failing, were found to be approximately normal. The absolute quantity and tension of CO_2 were both usually a little higher than before the injection. This is easily explained by the diminished respiratory movements and retarded circulation. Artificial respiration reduced the excess to a small extent. As death approached, both the tension and the quantity of CO_2 contained in the blood increased greatly from the same cause.

Influence of Snake Venoms on the Germicidal Action of Serum.—Weir Mitchell has frequently pointed out that, as a general rule, the characteristic local extravasations of crotalus poisoning suppurate or become gangrenous, and that bodies of animals dead of rattlesnake poisoning putrefy with extreme rapidity. Feoktistow made the interesting observation also that the bloody effusions in the pleura and pericardium sometimes contain micro-organisms immediately after death; and that cultures of these organisms, when injected into healthy animals, produce no effect. Ewing repeated some of Nuttall and Buchner's observations on the germicidal power of serum, and instituted a series of comparative experiments with the plasma or serum of animals poisoned with crotalus venom; he found that, after poisoning with crotalus venom, the normal germicidal power of rabbit's serum for *B. anthracis* and *B. coli* was entirely lost.

Experiments made in Sydney with the venoms of *Notechis pseudechis* confirmed these results of Ewing. In 1902, Flexner and Noguchi made a further study of this action with the venoms of the cobra, moccasin, copperhead, and rattlesnake, in the light of facts which had been ascertained concerning the phenomena of bacteriolysis. They experimented with *B. anthracis*, *B. coli*, and *B. typhi*, and the serum of the rabbit, and shewed that the action depended upon the presence in venoms of bodies of the nature of anti-complement.

Morbid Anatomy in Man.—The number of carefully recorded autopsies is small. In cases of death from *cobra-bite* rigor mortis occurs as usual. The areolar tissue in the region of the bite is infiltrated with pinkish fluid, and the neighbouring vessels are injected. The blood is often fluid, and when examined by the microscope directly after death it presents no changes. The brain appears to be normal; but the veins of the pia mater are usually gorged with blood, and the ventricles often contain turbid fluid. The lungs are usually congested, and the lining membrane of the bronchi is intensely injected. The appearance of the kidneys varies from the normal to one of excessive congestion.

After death from poisoning by one of the *viperine snakes*, the region of the bite is the seat of intense œdema and extravasation of blood. If the poison be introduced into the subcutaneous tissue the underlying muscles are frequently disorganised and even diffluent from extravasation of blood in their midst. Hæmorrhages may also be found in any of the organs and along the alimentary tract. The kidneys are acutely congested or hæmorrhagic. The blood is fluid.

Autopsies in cases of bite by *Australian species of snakes* present, as a rule, the same appearances as those detailed in cobra-bite. The blood is almost invariably fluid, but may contain a few soft coagula. The lungs may be the seat of hæmorrhages; in cases in which blood has escaped from mucous tracts during life these surfaces are intensely congested and hæmorrhagic. The nervous system merely shows hyperæmia.

In the case of bites by viperine snakes, should the patient survive for some days the region of the bite is often the seat of a large slough or even of a rapidly advancing emphysematous gangrene. This local condition will of course depend upon the species of bacterium which has become implanted in the tissues already devitalised by the venom.

Symptoms of Snake-bite in the Human Subject.—We have already pointed out that venoms are very complex substances, each made up of several constituents of different physiological action, and that the dissimilarity in the effects produced upon man and animals by the different venoms is entirely due to the proportions in which the different groups of toxic substances occur in each poison. The symptoms, in cases of snake-bite, also vary somewhat according to the amount of venom injected and the site of injection, in so far as this latter influences the rapidity of the absorption of the venom. The physiological actions and symptoms produced by venoms can only be roughly classified into two groups corresponding to the colubrine and viperine families. So that it is necessary to consider separately the symptoms seen in cases of intoxication from the venoms of some of the more important species, the bites of which are dangerous to life.

Cobra-bite.—The first symptom is a sensation of burning pain, more or less severe, at the seat of inoculation. The spot soon becomes red, tender, and swollen. An interval of about half an hour usually occurs before the patient experiences any constitutional symptoms: he then feels intoxicated, sleepy, and weak in the legs; the weakness increases until he is unable to stand. Profuse salivation, paralysis of the tongue and larynx, with inability to speak or swallow, soon supervene. Nausea and vomiting are of frequent occurrence. The paralysis now becomes more general and decided; the patient lies on his back, incapable of movement; his breathing becomes slower and the respiratory excursions diminish; he appears to be conscious, but unable to express himself; the action of the heart is quickened, but of fair strength. At length the breathing ceases, with or without convulsions, and the heart soon stops. Up to or immediately preceding the respiratory cessation the pupil remains contracted and reacts to light. Should the patient survive the paralytic symptoms he returns rapidly into a state of complete health. There are occasional discharges of blood from mucous surfaces, but the urine never contains albumin.

Bite of Bungarus Cœruleus (krait).—There is no doubt that bites from this snake are extremely dangerous, and that a considerable percentage of the total deaths from snake-bite in India, especially in Northern India, is due to it. The symptoms seen in cases of krait-bite are somewhat similar to those already described in cases of cobra-venom intoxication. In a recent case in a soldier in India, of which we have notes, the chief symptoms observed were paralysis of articulation, embarrassed and stertorous breathing, and semi-consciousness. There was no local swelling or reaction at the site of the bite, which was on the ring finger. Death took place about eight hours after the bite.

Bite of Bungarus Fasciatus.—No authentic cases of bites from this snake have been described in the human subject. The symptoms, therefore, which would be met with can only be deduced from those which have been observed in animal experiments, especially in monkeys. If a large amount of this poison be injected directly into the circulation, intravascular thrombosis results and the symptoms observed are due to this pathological condition. As, however, this can practically never happen in nature we need not further discuss it. The ordinary cases may be divided into two classes; those in which death takes place within the first forty-eight or seventy-two hours after the bite; and those in which death is delayed for from six to twelve days. In the first class of acute nervous cases the symptoms are similar to those seen in cobra-venom intoxication. Death is due to paralysis of the respiratory centre. The local reaction is much less than in cases of cobra-bite, in fact little or nothing is to be observed at the site of the bite. In the second class we have, however, to deal with a condition which is never seen in cobra-venom poisoning, nor in fact in poisoning with any other venom with which we have worked. In these cases death is delayed for from six to twelve days. There is as a rule an interval after the bite, varying from two to six days, in which no symptoms are observed. After this interval a comparatively chronic disease, which almost invariably ends fatally, begins. The symptoms which arise during this late period of intoxication are not in the least like those seen in the acute cases of poisoning, either with cobra-venom or with the venom of *Bungarus fasciatus* itself. There are loss of appetite and great depression ; but marked muscular weakness and atrophy, extreme emaciation, and loss of weight are the prominent symptoms. There is also a marked diminution of the urine, and irregular elevations of temperature are sometimes observed. Purulent discharges from the eyes, nose, and rectum occur late. The emaciation, loss of weight, and muscular atrophy progress rapidly, and, as a rule, end in death after a few days of illness.

Bite of Enhydrina Valakadien.—This species is the commonest of the sea-snakes ; it is very abundant along the coasts of India and Burma to the Malay Archipelago and New Guinea. There are no authentic cases on record of bites in the human subject, so in this instance also the description of symptoms is taken from animal experiments. The symptoms observed in animals after injection of this poison are very similar to those of cobra-venom intoxication. The local reaction is, however, very slight ; and, further, there are no symptoms which point to any action of the poison on the coagulability of the blood-plasma or on the red cells. There is progressive paralysis accompanied by dyspnœa, which latter symptom is much more marked than in cases of cobra-venom poisoning ; death is preceded by respiratory convulsions. The heart goes on beating for several minutes after the respiration has ceased.

Bite of Rattlesnake.—The pain of the wound is severe, and is speedily followed by swelling and discoloration ; sometimes there is hæmorrhage from the wound. Constitutional symptoms occur, as a rule, in less than fifteen minutes. These consist in progressive prostration, which eventually becomes appalling, staggering gait, cold sweats, nausea and vomiting, quick and feeble pulse, dilated pupils, and slight mental disturbance. In this state the patient may die about twelve hours after the bite. If he recover from the depression the local symptoms begin to play a much more important part than in cobra-poisoning. The swelling and discoloration extend up the limb and trunk, and symptoms of general blood-poisoning set in with rise of temperature, puffy face, great weakness, and repeated syncope. The pulse is quick and feeble, and the respiration laboured. Sometimes the mind is clear ; sometimes there are restlessness and delirium. Death may occur in this stage, and is sometimes preceded by convulsions. The local hæmorrhagic extravasation frequently suppurates or becomes gangrenous, and from the results of this the patient may die even weeks afterwards. When the dose has been a less severe one the swelling may decline and pain disappear rapidly. Recovery from the acute symptoms is in almost every case sudden and astonishing when contrasted with the gravity of the symptoms ; within a few hours the patient, from being in a moribund condition, becomes bright and intelligent.

Bite of the European Viper.—The symptoms following the bite of the European viper resemble those of a small dose of rattlesnake venom. The bite is immediately followed by local pain of a burning character ; the limb soon swells and becomes discoloured, and within from one to three hours great prostration, accompanied by vomiting and often diarrhœa, sets in. Cold, clammy perspiration is usual. The pulse becomes extremely feeble, and slight dyspnœa and restlessness may be seen. In severe cases, which occur mostly in children, the pulse may become imperceptible and the extremities cold; the patient may pass into coma. In from twelve to twenty-four hours these severe constitutional symptoms usually pass off; but in the meantime the swelling and discoloration have spread enormously. The limb becomes phlegmonous and occasionally suppurates. Within a few days recovery usually occurs somewhat suddenly, but death may occur from the severe depression, or from the secondary effects of suppuration.

Bite of Daboia Russellii.—This snake may be taken as the typical Indian viper; it is very widely distributed throughout the Peninsula, Ceylon, Burma, and Siam. It is also found in the Himalayas up to 6,000 feet at least. It is one of the most deadly snakes, and next to the cobra probably causes more deaths in India than any other snake. If daboia-venom be injected directly into the blood-stream, a condition which might happen in the case of a bite in man, violent convulsions rapidly set in and soon end in death. These symptoms are due to a more or less general intravascular thrombosis. When the poison is introduced into the subcutaneous tissues the symptoms may be divided into local and general. Locally there are very severe pain and rapid onset of swelling, which soon extends up the limb, if the bite is on an extremity; there is often a blood-stained discharge from the wounds. Ecchymosis is soon very apparent all round the site of the punctures.

The general symptoms are marked collapse, a small thready pulse, cold sweats, nausea, and vomiting, pupils dilated and insensitive to light, and often complete loss of consciousness. The patient may temporarily recover from these general symptoms, only to fall into a deeper state of collapse than before. Death often takes place within a short time after the infliction of the bite. If, however, recovery from this depression takes place the local condition plays an important part in the subsequent history of the case. The swelling extends rapidly; there is a large extravasation of blood with much œdema all round the punctures. Extensive local suppuration and sloughing, malignant œdema, or tetanus may supervene; and the symptoms of these conditions may now be added to those due directly to the venom. These latter symptoms consist of œdema of the dependent parts, and hæmorrhages, often severe, from the rectum and other orifices of the body; albuminuria or hæmorrhage from the kidneys is a constant symptom. Rapid emaciation soon appears, and in the prolonged cases a profound anæmia and lethargy set in. There is an absence of paralysis and of any symptoms which might point to any direct action of the poison on the central nervous system. Death may be delayed for several days; this, however, depends more on the local condition. Recovery in these cases is not at all uncommon.

Bite of Echis Carinata.—This is a comparatively small snake, although its poison is extremely active. The symptoms following its bite are similar to those of daboia-bite. The local swelling and hæmorrhagic extravasation are severe. As the amount of venom injected at each bite is comparatively small, recoveries are fairly common, and it is probable that the mortality from this snake is not large. Recently an authentic case came under observation in St. George's Hospital, Bombay, of which we have full notes. A man was bitten on the temple by an *Echis carinata*, which was in captivity in the Museum of the Bombay Natural History Society. He came under observation a quarter of an hour after the bite, and was then very frightened and had an anxious look on his face. The whole of the temple, on which two small punctures could be seen, was swollen and ecchymosed, the swelling extending to the side of the face and including the upper and lower eyelids. There was severe swelling over the wounds. The blood which exuded from incisions made over the punctures was very liquid, and remained unclotted. Vomiting soon began and continued till death. The pulse was very small, feeble, irregular, rapid, and at times could hardly be felt at the wrist. Extreme restlessness and complete insomnia were marked symptoms. The extremities were cold and clammy. The patient remained conscious for many hours; but a short time before death, which took place twenty-five hours after the bite, he became unconscious and delirious. There were no hæmorrhages from any of the orifices.

Bite of Australian Species of Snakes.—The pain and local swelling which follow a bite by one of the larger Australian colubrines are not usually severe. Constitutional symptoms appear in from fifteen minutes to two hours. The first symptom is almost invariably a feeling of faintness and an irresistible desire to sleep. On an attempt to walk the gait becomes staggering, and the weakness in the legs increases until the patient is unable to stand alone. Alarming symptoms of prostration then supervene, and are often accompanied by vomiting. The heart's action becomes extremely feeble, and the pulse thread-like and uncountable; the extremities are cold and the skin blanched. The respiration, which at first is somewhat quickened, becomes shallower from hour to hour as the coma increases. Sensation is blunted, and eventually stimulation of the nerves of special sense ceases to evoke any reaction: the pupil is widely dilated and insensible to light. In this state death may occur from gradual cessation of respiration; it is sometimes preceded by convulsions. The heart, which has become extremely feeble in its action, may sometimes be felt to beat for a few seconds after the cessation of respiration. In some cases hæmorrhagic extravasation also occurs from some mucous surface; so that the patient coughs or vomits blood, or passes it by the rectum or kidneys, but this is unusual. Albuminuria has been generally found when looked for, and may be accompanied by blood or blood-pigment in the urine. If the patient survive the coma, recovery is complete; and, as a rule, is rapid and without any secondary symptoms.

Prognosis.—If we assume that the susceptibility of man to snake-poisons is weight for weight not greatly different to that found for a monkey or a dog, then by reference to the tables above, setting forth the quantity of venom which different snakes can eject at one time and their minimal lethal dose, it is obvious that the larger serpents can discharge many lethal doses, in the case of the cobra 20 lethal doses, into a person bitten. Nevertheless, the mortality in persons bitten by the larger snakes of India and South America would not, from the scanty records available, appear to be more than 30 per cent. If these figures represent the truth, either the susceptibility of man, weight for weight, is greatly less than that of most other animals, or else in the circumstances in which a snake bites a man the reptile is seldom able to inject its full charge. The former alternative seems to us unlikely on general grounds, and we incline to the latter view as the more probable. When a snake bites a man, it is generally in a hurry and it is not permitted to gnaw away at its victim in the same way as it is encouraged to bite a watch-glass for the purpose of collecting venom in the laboratory.

Treatment of Cases of Snake-bite.—When a person has been bitten by a poisonous snake our efforts to influence the result should take two directions; (1) to prevent the absorption of the poison; (2) to counteract or lessen its effects on the organism.

(1) With the first object a ligature should immediately be placed on the limb above the situation of the bite; the ligature must of course be applied where there is only one bone and not in the forearm or lower leg. Wall strongly recommends a stout india-rubber band as being by far the best material for use as a ligature; but in ordinary circumstances only a portion of clothing would be available, and answers quite well. The ligature must be tightly applied; this is easily attained by passing a stick under the ligature and twisting it. The action of the ligature is to delay the absorption of the venom for just so long as it may be applied, and in the case of venoms, such as those of the cobra and the krait, which contain no fibrin-ferment, this is all that is accomplished by its use. In those venoms, however, which, like *Echis carinata* and many of the Australian snakes, contain fibrin-ferment, it operates in another way and one which would hardly have been expected. Experiments with the poison of *Notechis scutatus* and *N. pseudechis* have shown that six times a fatal dose of these venoms may be injected into the leg of a rabbit and be followed by little or no constitutional disturbances, provided an elastic ligature be applied immediately afterwards above the seat of inoculation and allowed to remain on for twenty minutes. These experiments have been frequently repeated, and on dissecting down into the situation of the injection it was discovered that the blood and lymph in the immediate neighbourhood of the seat of inoculation were completely solid, so that the poison was temporarily shut up and could only be slowly absorbed subsequently. Moreover, it is not unlikely that a good deal of it became combined with the cells in the locality. On repeating these experiments with the same venoms which had been exposed to a temperature of 75° C., so as to destroy the fibrin-ferment, the only result of ligaturing the limb was to delay the onset of symptoms and death of the animal for so long as the ligature had been applied.

Having by means of the ligature insured that no more poison is absorbed, the next step is to destroy or remove what poison still remains deposited locally, with the object of preventing as much as possible of the venom from entering into the general circulation on removal of the ligature. There is great difficulty in thoroughly accomplishing this object. Wall has correctly pointed out that the poison is deposited not in the skin itself but in the areolar tissue beneath, and that as the skin is as a rule freely movable over the parts below, the fangs may have dragged it away from its proper position before the poison is injected. In this way it may happen that the poison is not deposited immediately beneath the punctures. Keeping this possible contingency in view, Wall strongly recommends careful and deep dissection with the knife of all the parts likely to contain the poison. The dissection must be free in all directions, especially so in the direction of the lymphatic and venous return. In the case of the fingers, hand, and such parts it should be carried right down to the bone. After this free and careful dissection, the wound should be freely washed out with a strong solution of permanganate of potash, which will destroy any poison with which it comes in contact. Many other methods have been recommended for the destruction of the poison locally. The injection of any substance, such as bleaching powder or chloride of gold, as recommended by Calmette, can be made by guess-work only, as we do not know exactly where the poison lies; and the solution, instead of following the venom, will take the line of least resistance in the tissues, and if the patient recover will produce a nasty slough. Washing the punctures with any kind of solution is of course futile. Wall also considers that the application of any destructive agent to an incision through the wounds is almost useless; he found that such a method sometimes succeeded after the injection of the poison with a syringe, but was without avail after the natural bite of a snake. The same difficulty will be met in the method lately advocated by Sir L. Brunton, Sir J. Fayrer, and Major Rogers, in which it is advised that an incision be made over the punctures with a lancet-shaped blade and crystals of permanganate of potash then be rubbed in. Major Rogers found this method successful in the case of cats, but in these experiments he knew accurately the direction taken by the point of the needle; in practice we fear that it will only be by chance that the permanganate reaches the poison, which may be placed deeply in the areolar tissue and at some distance from the punctures. The method, therefore, must be much less efficacious than that of free dissection, as recommended by Wall, followed by swabbing out of the wound with a strong solution of permanganate of potash. To suck the wounds is absolutely useless.

(2) As well as attempting to remove and destroy as much as possible of the venom which has been deposited locally, we must endeavour to render innocuous any poison which has been already or is likely to be absorbed into the general circulation. Various remedies have been suggested and strongly recommended for the treatment of cases of snake-bite. Alcohol, ammonia, and strychnine have been strongly advocated; the two latter especially in Australia. But all experimental

work, as well as practical experience, prove that none of these medicines is of the slightest use as an antidote to snake-venom. In India numbers of quack medicines are year after year recommended, only to be found quite useless when tested experimentally. It is quite unnecessary to consider these remedies.

The Serum Treatment of Snake Poisoning.—While all these empirical methods have failed us in the treatment of cases of snake-bite, recent research has shewn that along the line of serum therapeutics lies the true antidote for snake-venom intoxication. In 1894 Calmette established that an animal that has been treated over a length of time with the venom of a poisonous snake, such as the cobra, yields a serum which is antitoxic towards that poison. On account of the interest and importance of the subject, a short description of the work which has led up to this result will not be out of place.

The belief that by the repeated injection or eating of snake-poisons some amount of immunity can be established, has been current from very ancient times amongst the inhabitants of many lands where poisonous serpents abound. Sir T. R. Fraser has collected a number of these legends and presented them in an interesting introduction to his lectures on Immunisation against Serpent's Venom, delivered at the Royal Institution (*Nature*, April 23, 1896). That individuals who have repeatedly been bitten by snakes, such as some native snake-charmers and others in the habit of manipulating these reptiles, may possess a considerable amount of resistance, is undoubted. We are aware of the case of a European in Australia who used to manipulate *Notechis scutatus* with impunity, and who was bitten on more than one occasion in our presence, with but slight manifestation of ill effect. This individual was, however, under the impression that his immunity was equally valid for other poisonous species, and on being bitten by a large specimen of *Denisonia superba*, in endeavouring to secure it, died on the following day.

Immunity against snake-venom by experiments upon animals was first obtained by Sewall, who shewed that repeated injections of small doses of rattlesnake poison establish a resistance to the poison. Subsequently Kanthack succeeded in obtaining some resistance to cobra poison, but was unable to obtain complete immunity. Phisalix and Bertrand in 1893 induced some immunity in rabbits by repeated injections of the European viper's venom, which had previously been heated to 80° C., and found that the serum of the animals thus treated was possessed of curative properties. In 1894 Calmette published an account of his extended researches on this subject. By patiently immunising horses against cobra-venom, Calmette succeeded in producing sufficient antitoxin in their serum to be of some practical value. In the following year Sir T. R. Fraser, who had also been investigating the same problem, published a confirmation of Calmette's results and some further points of importance in relation to the nature of the protective action of the serum. The results of these two observers, working quite independently of each other, shewed that a solid immunity could be obtained by repeated, long-continued injections of snake-venom, and that the serum of an animal treated in this way contained antitoxic substances. Calmette then proceeded to produce an anti-venomous serum in sufficient quantity for therapeutic purposes. This serum was prepared with a mixture of snake-venoms, in which mixture, however, cobra-poison greatly preponderated.

The Specificity of Anti-Venomous Serums.—Calmette asserted that the serum prepared at Lille was active against the venoms of all species of snakes, a claim based on the conception that all snake-venoms are alike in physiological action and differ from one another only in their degree of toxicity. In the light of more recent research, this conception is, as we have seen, no longer tenable, and the contention regarding the universal applicability of the serum prepared at Lille has been shewn to be without foundation. This serum was found inadequate to preserve animals against injections of the poison of the Australian tiger-snake, although it possessed a certain neutralising value for the neurotoxin of this poison. Subsequent experiments in Sydney shewed that the same was true as far as the poisons of other Australian snakes were concerned. In India it was shewn that the Lille serum, while active for cobra-venom, had no neutralising effect for the poisons of the following snakes, namely, *Daboia russellii*, *Bungarus fasciatus*, and *Echis carinata*.

Recently Calmette went so far as to admit that in some venoms there are two poisonous constituents, namely, a neurotoxin and a hæmorrhagin; and that the antitoxic serum prepared by injecting cobra-venom is without action upon this hæmorrhagin, but that such a serum is distinctly antitoxic to all the neurotoxins of other venoms. This hypothesis, however, is not in consonance with the facts which have been acquired by the careful testing, both *in vivo* and *in vitro*, of various serums prepared with pure unmixed venoms.

As this question of specificity or non-specificity of serums is of practical importance, results which have been obtained with six pure anti-serums are worthy of mention in some detail: (1) a serum prepared by Tidswell with the poison of *Notechis scutatus*; (2) a serum prepared by one of us [G. L.] with cobra-poison; (3) a serum prepared by one of us [G. L.] with the venom of *Daboia russellii*; (4) a serum prepared by Noguchi with crotalus venom; (5) a serum prepared by Noguchi with moccasin poison; (6) a serum prepared by Brazil with Lachesis poison.

(1) Serum prepared with the poison of *Notechis scutatus.*—This serum tested *in vivo* has been shewn to be strongly antitoxic for its homologous venom, but to be without neutralising action for the venoms of *Pseudechis* and *Diemenia*, two species of Australian snakes, the poisons of which have physiological actions apparently identical with those of the venom of *Notechis scutatus.* It has also been shewn to be without neutralising effect on the general actions *in vivo* of the poisons of the following colubrine species: Cobra, king cobra, *Bungarus cœruleus*, *Bungarus fasciatus*, *Enhydrina valakadien*; and of the following vipers: *Daboia russellii*, *Echis carinata*, *Lachesis gramineus*, and *Crotalus durissus.* When this serum is tested *in vitro* against the hæmolytic action of the several venoms, the following result is obtained: it neutralises the venom of *Enhydrina valakadien* rather better than it

neutralises the poison with which it has been prepared; also, it has a marked inhibitory effect on the action of cobra-venom and of *Echis carinata* poison; but it has no inhibitory effect on the hæmolytic action of other six poisons, namely, the venoms of the king cobra, of *Bungarus cœruleus*, of *Bungarus fasciatus*, of *Daboia russellii*, of *Lachesis gramineus*, and of *Crotalus durissus*.

Finally, this serum has been tested against the clotting action which several venoms exert on citrate plasma, that is to say, against the fibrin ferment contained in the poisons. The results of these observations shew that, while the serum neutralises well in its homologous venom and also that of *Notechis pseudechis*, it is quite inactive against three other poisons, namely, the ferment in the venoms of *Echis carinata*, of *Lachesis gramineus* and of *Crotalus durissus*.

(2) Serum prepared with pure cobra venom.—This serum, tested *in vivo* against the general actions of various poisons, is found to be strongly antitoxic for the venom used in its preparation; when used in large quantity it has a slight neutralising power for the venom of *Enhydrina valakadien*; further, it delays death in cases of intoxication with the venoms of the king cobra and of *Bungarus fasciatus*, but even when used in large quantities does not completely neutralise these poisons. This serum contains no antitoxic substances active against the venom of *Bungarus cœruleus*. It was also found to be without neutralising effect for the venoms of the following viperine snakes: *Daboia russellii*, *Echis carinata*, *Lachesis gramineus*, and *Crotalus durissus*.

Cobra anti-venom was found to be largely specific as far as the hæmolytic actions of the several venoms are concerned: thus it neutralises its homologous venom in a marked degree; it prevents to a certain extent the hæmolytic action of the venom of *Bungarus cœruleus*, a relatively much greater quantity of serum, however, being required to effect this than in the case of cobra venom; finally, it has no hindering effect at all on the hæmolysing action of eight other poisons, namely, the venoms of king cobra, of *Bungarus fasciatus*, of *Notechis scutatus*, of *Enhydrina valakadien*, of *Daboia russellii*, of *Echis carinata*, of *Lachesis gramineus*, and of *Crotalus durissus*. The serum was further tested, also *in vitro*, against the anti-clotting action of both cobra and king-cobra venoms. It was found to neutralise well the venom of the cobra, but to have little or no effect on king-cobra poison.

(3) Serum prepared with the venom of *Daboia russellii*.—This serum has also been tested both *in vivo* and *in vitro*. When tested *in vivo* against the general actions of various poisons, it was found to have no effect whatever on any of five colubrine venoms, namely, the venoms of the cobra, king cobra, *Bungarus cœruleus*, *Bungarus fasciatus*, and *Enhydrina valakadien*; to neutralise well its homologous venom; to have a certain but not very marked neutralising effect on the venom of another viper, namely *Crotalus durissus*; to have no antitoxic action for the venom of a closely allied viper, *Echis carinata*, nor for that of another Indian viper, *Lachesis gramineus*. When tested *in vitro* against the hæmolytic actions of several venoms, it was found that this

serum has no hindering effect whatever on the venoms of six colubrine snakes; that it neutralises equally well the venom with which it was prepared and the venom of *Echis carinata*; that it has a considerable but less neutralising effect on the venom of *Crotalus durissus*, but no hindering effect on the poison of *Lachesis gramineus*. Further, when tested *in vitro* against the fibrin-ferment actions of various poisons, it was found that, while the serum neutralises well its homologous venom, it has no effect on the poisons of four other species, one colubrine and three viperine, namely, *Notechis scutatus*, *Echis carinata*, *Lachesis gramineus*, and *Crotalus durissus*.

(4) and (5) Serums prepared with crotalus venom and with moccasin venom.—The details of the testing of these two serums have not been published in a complete and full form; but the data which are available are clearly of the same nature as those obtained with the three serums mentioned above.

(6) Brazil found that the Lille serum was unable to save animals poisoned with lachesis and crotalus venoms; that his serum prepared with the venom of lachesis was without action upon crotalus poison, but that the anti-crotalus serum did possess some action against the venom of *Lachesis lanceolatus* as well as that of crotalus.

All these careful and detailed observations point to the same conclusion, namely, that both in *in vivo* and *in vitro* anti-venomous serums are highly but not strictly specific.

The neurotoxin of cobra venom is evidently somewhat different from that of the king cobra and *Bungarus fasciatus*, and quite different from that of the common krait (*Bungarus cœruleus*). Further, it is also clear that the hæmolytic constituents, the hæmolysins, differ markedly from one another. The same can be said of the fibrin-ferments of the various venoms. In short, Calmette's conception of the identity of neurotoxins, etc., is not justified by the facts; on the contrary, it is clear that identity in physiological action does not justify the assumption that a serum prepared with one venom will neutralise another. Megatheriolysin and tetanolysin both dissolve corpuscles, but a serum prepared against one is without effect upon the other.

The bearing of this conclusion on the problem of the serum-therapeutics of snake-bite is evident, for at the outset we are met with the almost insurmountable difficulty that only the specific anti-serum will be of use in a case of snake-venom intoxication. Now, in the first place, it seems almost impossible to collect these various venoms in quantities sufficient for the purpose of immunisation of large animals; and, in the second place, granted that it were possible to prepare anti-serums for the different poisons, the practical use of these would be beset with difficulty. For when a person is bitten by a snake he is rarely able to tell the species of snake which has inflicted the bite; furthermore, as an anti-venomous serum, to be of much practical utility, must be injected before any symptoms of intoxication have set in, the medical man who is called on to treat a case of snake-bite is not as a rule in a position to form an opinion, either from the history of the case or from the symptoms, as to the nature of the venom which has been injected. Probably the solution of this difficulty will lie in our being able ultimately to prepare a polyvalent serum with the venoms of the commonest and more deadly snakes. This principle is carried out to a limited extent at Lille ; and from the Pasteur Institute of India a serum is being issued which is prepared by immunising horses with the unheated poisons of the cobra and the daboia, the two snakes chiefly responsible for the 20,000 or more deaths which occur annually from snake-bite in India. So far it has been found impossible to procure enough venom from the other Indian species to immunise large animals.

The Dose of Serum Necessary.—Calmette considers that 10 c.c. of the Lille serum is sufficient in most cases of snake-bite ; but that if the treatment is delayed, or if the snake is a cobra or a krait, 30 c.c. should be used. He recommends that the injection should be given subcutaneously unless symptoms are already present, in which case an intravenous injection of 10 or 20 c.c. is required.

For reasons which will appear below, we are convinced that these small quantities are quite insufficient in the case of the cobra, against which the Lille serum is particularly antitoxic. In order to arrive at an accurate therapeutic dose of an anti-venomous serum three factors must be determined: (1) the neutralising power of the serum, that is to say, the exact amount of serum which can neutralise a given amount of venom ; (2) the amount of poison which the snake can inject ; (3) the quantity of venom, the injection of which a man can survive.

Two c.c. of the Lille serum are stated to have the power of neutralising 1 milligramme of dried cobra-venom, and the serum issued by the Pasteur Institute of India is double this strength, or 1 c.c. neutralises 1 mg of cobra-venom. If this is expressed in terms of the number of fatal doses for a guinea-pig of 250 grammes which can be neutralised by 1 c.c. of serum, a comparison may be drawn between the relative potency of anti-venom and the antitoxins for diphtheria and tetanus. Expressed in this way, 1 c.c. of the Indian serum neutralises only 10 minimal fatal doses for a guinea-pig of standard weight. On the other hand, 1 in every 4 horses will produce a diphtheria antitoxin, 1 c.c. of which, when tested under the same conditions, will neutralise 100,000 minimal fatal doses of toxin, or, if immunised against tetanus, will furnish tetanus antitoxin, 1 c.c. of which will neutralise 200,000 minimal fatal doses. It will be seen at once that, compared with these antitoxins, anti-venom is very weak indeed ; in fact, if the other antitoxins were only possessed of such small neutralising power they would be of little practical use. It has not at present been found possible to immunise a horse so that it shall produce a serum of greater power, but possibly, by different methods of immunisation, serums of greater potency may be produced in the future.

We have thought it advisable to lay stress upon the real antitoxic value of anti-venoms, because we think this point has been very imperfectly realised.

The second point, namely, the amount of venom which a snake can inject, has been settled in the case of cobra and daboia venoms by actual experiment. In the cobra this amount varies from 200 to 350 milligrammes of dried venom, and in the case of the daboia it is somewhat less, namely, 150 to 250 milligrammes. These observations were carried out on fresh snakes recently caught. Calmette has made a series of experiments with cobras which had evidently been kept in captivity for some time; the greatest amount of dried venom which he was able to procure was 48 milligrammes, and the average amount was about 20 milligrammes The therapeutic dose of serum recommended by him is calculated on this basis, and is evidently therefore much too small. It is obvious that in estimating the dose of anti-venomous serum to be used in the treatment of any case, say of cobra-bite, it should be borne in mind that the snake *may* have injected as much as 350 milligrammes, for the neutralisation of which quantity sufficient serum must be injected in order to save the patient's life. There must be, of course, many cases in which less, probably a good deal less, venom would be injected, and a smaller amount of serum would then suffice; but there are no means by which this point can be estimated, and as treatment must be immediate there is no time to wait and watch the development of the case.

The third factor, the amount of venom which a man can survive, must next be considered. This can, of course, only be estimated roughly from experiments on animals. Basing our calculations on the results obtained with monkeys, we have arrived at the conclusion that for an average man the lethal dose of cobra-venom would be about 15 to 20 milligrammes, and of daboia-venom about 60 milligrammes. A man would, therefore, be able to survive slightly less than these amounts of poison.

Having as far as is possible arrived at the data on which the dose of serum to be used in any case of cobra or daboia-bite rests, we have now to calculate what this dose ought to be. In the case of a cobra-bite, supposing the serum to be of such strength that 1 c.c. is able to neutralise 1 milligramme of pure cobra-poison, if the snake has injected the maximum amount of poison, namely, about 350 milligrammes, we can calculate that, in order to neutralise this quantity of venom and thus save the life of the patient, 350 c.c. of serum would have to be brought in contact with it. In the case of a daboia-bite a much smaller quantity of serum would serve the same purpose, as we have seen that this snake does not inject so much poison as the cobra, that the serum neutralises about double the amount of daboia-venom, and further, that the minimum lethal dose of daboia-venom by subcutaneous injection is much greater than that of cobra-poison. We can therefore calculate that about 100 c.c. of serum would be sufficient to neutralise the amount of venom which a full-sized daboia would be able to inject.

We have still to consider the method of injection, as these quantities of serum are the amounts which would be required to neutralise completely the venoms when they are mixed *in vitro* before injection. It has been shewn that as good results follow the simultaneous injection of a quantity of venom subcutaneously and of a neutralising dose of anti-venom intravenously into an animal as are obtained when the venom and serum are mixed *in vitro* before injection. If, however, the serum be injected subcutaneously at the same time as the venom, it was found that from 10 to 20 times the neutralising dose of serum was required to save the life of the animal. Applying these observations to our present purpose, we arrive at the conclusion that in treating a case of cobra-bite in which the snake had injected the maximum amount of poison, we should have to inject 350 c.c. of serum intravenously or from 3500 to 7000 c.c. subcutaneously in order to save the life of the patient (!), and in the case of daboia-bites 100 c.c. intravenously or from 1000 to 2000 c.c subcutaneously (!). Further, if symptoms have already appeared before treatment was begun, larger amounts of serum would be required to dissociate the poison from its junction with the cells. If the Lille serum be employed these quantities must be doubled, as it is only half the strength of that issued by the Pasteur Institute of India.

We have no desire to discourage the use of anti-venomous serums, for once the poison has been absorbed they are the only remedies available; but we wish to point out what is the real neutralising value of the serum and what may reasonably be expected from its use, and, further, to emphasise that quantities of 10 to 30 c.c., as recommended by Calmette, are hopelessly inadequate and only likely to lead to disappointment.

There are, of course, many cases of snake-bite in which for many reasons the snake does not inject anything approaching 300 milligrammes of poison, and for such cases treatment with a much smaller quantity of serum than those mentioned above would suffice. It is in fact probable that the great majority of cases would come into this latter category; but it must always be remembered that if the larger amounts of serum are not injected the treatment may fail because more than a lethal dose of poison remains unneutralised.

In the case of the bite from a snake for which a specific serum is available, *e.g.* cobra or daboia, this should always be injected. For reasons which have been set forth above, the following instructions should be followed:—

(1) The injection should be made as soon after the bite as possible.
(2) The injection should always be made intravenously.
(3) At least 100 c.c. of serum should be injected.

In cases of bites from species of snake for which no specific serum exists, no benefit can be hoped for from the use of any other serum. As, however, it is extremely rare to be able to identify without any doubt the species of snake which has inflicted the bite, the serum treatment should be employed in India in every case of snake-bite, on the chance that the snake was either a cobra or a daboia.

We are still far from the goal of an efficient polyvalent serum. It would be of the utmost value if we were able to obtain a stronger serum in a short interval of time without causing injury to the animals producing the serum. All workers on this subject are agreed that in order to procure a serum of sufficient strength for practical therapeutic purposes horses have to be treated over a period of from one year to a year and a half. Further, after each injection an abscess nearly always forms, and sometimes there is most extensive sloughing. By modifying venoms so as to produce toxoids prior to injection, much perhaps may be done to obviate these objections. We have already mentioned that Flexner and Noguchi were able to immunise rabbits with crotalus venom which had been modified by treatment with weak hydrochloric acid. The solution of the problem of obtaining a serum which is efficient for most species of snakes is also hampered by the difficulty of obtaining supplies of poison sufficient for the immunisation of large animals. Thus in India, under the very best conditions and with the strong support of the Government of India, it has been found impossible to obtain any venoms, with the exceptions of those of the cobra and the daboia, in sufficient quantity to immunise animals. Until this difficulty has been overcome no progress can be made in this direction.

As regards the treatment of snake-bite other than by anti-venomous serums, little or nothing can be done beyond keeping the patient quiet and *warm*. Small doses of alcohol may no doubt be beneficial as a stimulant but this end can be more effectually gained by ammonia or strychnine, and the employment of enormous doses of alcohol cannot receive too strong a condemnation. Major L. Rogers has recommended the use of adrenalin chloride in cases of bites from daboia and those snakes the poisons of which have a marked paralytic action on the vasomotor centre. On physiological grounds this drug should no doubt be of service in these cases. We are not aware that it has ever been used in practice.

To exhaust the patient when in the lethargic stage by walking him about incessantly cannot but be detrimental to his chances of recovery.

In conclusion, we have again to impress upon all who are called on to treat cases of snake-bite that every endeavour must be made to prevent absorption. An efficient ligature should be applied at once; then free dissection around the punctures, especially in the direction of the lymph and venoms return, should be practised, the wound being swabbed out with a strong solution of permanganate of potassium. These measures can usually be undertaken immediately, and in the present state of serum-therapeutics against snake-venoms more reliance should be placed on this local treatment than upon the injection of any anti-venomous serum.

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Investigations on the Development of Trypanosomes in Tsetse-Flies and other Diptera.

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With Plates 8—13 and two Text-figures.

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I. INTRODUCTORY.

IN this memoir I propose to give a full account of the work done and the results obtained by me during my stay in Entebbe, Uganda, as a member of the Sleeping Sickness Commission from the beginning of April to the beginning of

December, 1905. Some of my results have already appeared in the form of preliminary reports; but with regard to some few points further study necessitates revision or correction of statements previously made. In my eight months in Uganda I accumulated a great deal of material which it has required much time and labour to work out. The study of this material has occupied the time left from other duties since my return from Uganda, and may now be considered complete.

The commission with which I went to Uganda was to study the life-cycle of Trypanosoma gambiense in its relation to the local species of tsetse-fly, Glossina palpalis. In view of the well-known life-cycle of the malarial parasite, as well as of the remarkable development described by Schaudinn (**40**), for the trypanosomes of the little owl (Athene noctua), it seemed probable that a study of the sleeping sickness trypanosome in its passage through the tsetse-fly would reveal an interesting developmental cycle. Unfortunately, these expectations have not been fulfilled, and, so far as the development of Trypanosoma gambiense is concerned, my investigations have been barren of results, and have yielded conclusions for the most part of a negative character. Incidentally, however, I have made some observations which are, perhaps, not without interest, on another species of trypanosome occurring in the fly, and a record of my work and its results may be of some use if only as a guide to future investigators in this field to enable them to avoid my failure.

In the exposition of the numerous and complicated data furnished by an investigation of this kind, it is difficult to steer clear between, on the one hand, too much subjective interpretation, which may become misleading; and, on the other hand, excessive elaboration of detail, which becomes tedious and difficult to follow. I propose, therefore, to divide this memoir into two chief parts: I shall first set forth the development, so far as I have observed it, of the two species of trypanosomes in a connected manner, and shall discuss the general question of the transmission of trypanosome infections; after which I shall give a bare record, in chronological sequence, of my experiments and observations. The latter part is intended rather for reference, and to avoid the possible danger of omitting something which in the present state of our knowledge seems immaterial, but which may prove in the future of greater importance.

Before proceeding to describe my results it may be permitted to me to enter upon a brief personal explanation of my relation to the work of the Commission; after which I shall say a few words upon the methods of investigation employed.

When I came to Entebbe, at the beginning of April, 1905, my two colleagues, Lieutenant A. C. H. Gray, R.A.M.C., and the late Lieutenant F. M. G. Tulloch, R.A.M.C., were already at work upon the subject which I was sent out to investigate, and had discovered some facts of importance. In particular, they had found and studied the vast swarms of trypanosomes which are frequently found in the alimentary canal of freshly-caught tsetse-flies. Neither my colleagues nor myself had at that time any doubt but that these "wild" trypanosomes represented stages of T. gambiense. Since it was arranged that Gray and Tulloch were to work in connection with me, and since I did not wish to absorb, as it were, into our joint work anything of which the credit belonged to them independently, I requested them to write up and publish all that they had found before my arrival, so that we could start our collaboration on a clear footing. This they did, and the result was the memoir (**17**) published in the 'Sleeping Sickness Reports,' a memoir in which Tulloch's excellent drawings were done scanty justice in the reproduction. While Gray and Tulloch were engaged upon this report I commenced a systematic investigation of the anatomy of Glossina palpalis, then an untrodden field of study, upon which a preliminary report was published in the 'Proceedings of the Royal Society' (**27**). One of the first results of my dissection of the fly was to show that the structure termed by Gray and Tulloch in their memoir "the salivary gland" was really the proventriculus, although I myself fell into the error of calling it the stomach in my communication.

Our further studies were divided between us as follows: Gray and Tulloch continued the investigation of the "wild" trypanosomes. I confined myself to a study of the changes undergone by T. gambiense when taken up by the tsetse-fly from infected animals. My intention was to work the development of T. gambiense from the beginning, so to speak, up to the condition found by Gray and Tulloch in the fresh-caught flies, and so link my researches on to theirs, with hope of thus getting the complete cycle. This expectation was not fulfilled, since more extended knowledge forced the conclusion slowly, but irresistibly, upon us, that the "wild" trypanosomes had nothing whatever to do with T. gambiense, but represented at least two distinct species occurring in the fly quite independently of sleeping sickness. As a result, however, of our subdivision of the work, my two colleagues accumulated an abundant material of the "wild" trypanosomes; my material of these interesting forms consists only of those found incidentally in flies used for experiments with T. gambiense, in all six flies having been found by me thus infected.

I returned to England at the end of 1905, leaving Gray and Tulloch still at work upon these problems. Our ever increasing doubts as to the true nature of the wild trypanosomes led to our planning a number of crucial experiments, which were carried out by Gray and Tulloch on the island of Kimmi after my departure. The result was to demonstrate conclusively the distinctness of the "wild" trypanosomes from T. gambiense. Meanwhile I had written, shortly after my return to England, an interim report on my work at Entebbe, for the information of the Tropical Diseases Committee of the Royal Society. This report was also utilised by me in my candidature for the chair of Protozoology, which I now hold, and a printed copy of it was sent by me to my friend M. Mesnil, of the Pasteur Institute, who, not being aware of its private nature, of which he had not been informed, inserted an abstract of it in the 'Bull. Inst. Pasteur.' I am indebted to M. Mesnil's happy indiscretion for securing to me the priority of the statement, that the "wild" trypanosomes of Glossina palpalis were quite distinct from those of sleeping sickness—a conclusion to which Novy came independently after studying preparations sent him by Gray; his results were published in a memoir (**32**), in which he named one of the wild species T. grayi.

Shortly after this came the appalling news that our colleague, Tulloch, had become infected with trypanosomes. This most sad event, of course, put an end to further work, and Gray returned to England, bringing Tulloch with him. We then wrote a preliminary account of our results (**29**), which, so far as poor Tulloch was concerned, was a posthumous work when published. Our collaboration then broke up, and I began the detailed study of my material, and discovered the encystment of T. grayi, a result which I at once communicated to the Royal Society (**28**). It is a common human weakness to attribute failures to bad luck; nevertheless I cannot but deeply regret that I did not discover the encystment while I was in Entebbe. Had I done so, it would have modified the whole course of my researches. The fly in which this discovery was made was one of the last examined by me, a fortnight before I left off working. As I was then professor at University College, however, it was imperative upon me to return to my duties, having already had my leave of absence generously extended by the College for an extra three months.

Such is, in brief, the history of the conditions under which these investigations were carried on. It only remains for me to perform the pleasant duty of thanking those who have helped in my task. To the Tropical Diseases Committee of the Royal Society, in the first place, I am indebted not only for sending me to Uganda, but for providing me with assistance in working through my material after my return. Without the help and experienced advice of my colleagues on the Commission, Gray and Tulloch, I could have done little.

Since my return to England, my two assistants, Dr. J. D. Thomson and Dr. H. M. Woodcock, have rendered me much service in various ways, and Miss E. Y. Thomson has been of great assistance in the tedious task of searching through blood-films for trypanosomes and making counts of them. To each and all of these I desire to express my obligations and return my best thanks.

Methods of investigation.—I commenced my work, as I have said, by making a study of anatomy of Glossina palpalis. In undertaking this I was influenced largely by Schaudinn's work (**40**) on Trypanosoma noctuæ, in which he describes extensive migrations of the trypanosomes in the body of the invertebrate host. In tsetse-flies, however, I never found trypanosomes outside the alimentary canal, in spite of much searching; in this point my results agree with those of Stuhlmann (**41**).

My report on the anatomy of the fly (**27**) contained some errors which I desire to correct; they have been corrected in the reprint in the Reports of the Sleeping Sickness Commission. All through I used the term stomach for what should have been called the proventriculus; in this memoir I shall use the latter term. The true stomach in Glossina is represented by the first part of the digestive tract in the abdomen, that is to say, by the coils which in my figures and description were numbered 5, 6, 7. It is this region which becomes congested with blood after feeding, and in which the blood retains its bright red colour. From the stomach the blood passes into the next coils of the digestive tract, which constitute the true intestine, and here it becomes blackish in colour. Hence we may conveniently speak of the red blood, meaning that in the stomach, and the black blood, meaning that in the intestine proper. The red blood is thick and jelly-like, very difficult to smear out unless broken up with salt-citrate solution[1]; the black blood, on the contrary, is very fluid and watery, the number of corpuscles is more or less diminished, and there are usually numerous square crystals in it. The black blood stops short at the point where the Malpighian tubules arise, and where the proctodæum commences. The proctodæum contains no blood, but a yellowish fluid in which are suspended numerous coarse granules showing Brownian movement. Hence in a fly about twenty-four hours after feeding the three regions of the abdominal gut, namely, stomach, intestine, and proctodæum, are marked out by their respective colour—red, black, and pale yellow—in a way to make them easily discernible with the naked eye.

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[1] Made up as recommended by Laveran and Mesnil (**23**); ·5 gr. sodium chlorine + ·5 gr. sodium citrate + 100 cc. H_2O.

In the examination of a tsetse-fly for trypanosomes I usually began by inserting a fine capillary tube into the pericardial space, in order to draw up some of the fluid circulating in the hæmocœle. It was usually possible to draw up a small drop of the fluid. Some flies, however, were very anæmic and dry; such flies always proved on further examination to be sickly, and usually contained great numbers of bacteria in the gut, in which no blood as a rule was found. The hæmocœle fluid contained always peculiar amœboid corpuscles, generally more or less fusiform, with each end prolonged into a pseudopodium-like process; sometimes one end would show two such processes. The corpuscles showed distinct changes of form. No trypanosomes, however, were found in the hæmocœle. The next step would be the removal of the dorsal integument of the abdomen, and the examination of the contents of the salivary glands and the Malpighian tubules. The salivary glands contain numerous corpuscles or spherules of circular contour, varying from about 9 to 15μ in diameter, imbedded in a granular matrix. Each corpuscle contains an irregular spot towards the centre, usually angular in shape and looking like a split or space. With picrocarmine the corpuscle takes a pink tinge and the central spot a deeper colour. In the fresh condition the salivary corpuscles are about the same size as the fat-globules liberated from the fat-body, but distinguishable from them by their less refringent appearance and also by the fact that the fat-globules float upwards, while the salivary corpuscles lie at the lowest focus. The Malpighian tubules contain coarse, yellowish-brown granules similar to those found in the proctodæum. Neither in the salivary glands nor in the Malpighian tubules did I ever find trypanosomes or any bodies other than those already mentioned. As I have already stated, the statement of Gray and Tulloch (**17**), to the effect that they found trypanosomes in the salivary gland must be corrected; I was present when they made the observation, and saw the preparation; by salivary gland was meant proventriculus.

After the Malpighian tubules I next examined the genitalia, either testes and seminal vesicles, or ovaries, receptacula, and oviducts (including uterus and embryo, if present). Here also I found in no case anything but the proper contents of these organs, which I need not describe in detail.

The last process is to dissect out the gut for its whole length and to divide it into its four regions, namely (1) the proventriculus with the thoracic intestine and sucking stomach, (2) the stomach with the red blood, (3) the intestine with the black blood, and (4) the proctodæum and rectum. In none of the flies dissected by me did I find trypanosomes in the proventriculus, but Gray and Tulloch, with their more abundant material of T. grayi and T. tullochii, frequently found these species in this part. No trypanosomes were ever found in the sucking stomach,[1] which in the normal condition is filled with air, but which immediately after feeding is found to contain traces of blood, and is sometimes quite full of blood, though this is a rare condition, and perhaps the result of some functional derangement of the organ. The fly, when feeding, appears to fill the sucking stomach with blood, and then to expel it thence into the digestive tract. It can be observed that when sucking blood the fly at intervals raises its head up a little, thus partially withdrawing the proboscis from the skin of its victim, and then lowers it again.[1] Probably when the proboscis is lowered the sucking stomach is being filled, and when it is raised the blood is being expelled from the sucking stomach. This would account for the traces of blood found in the sucking stomach, and also in the proventriculus and thoracic intestine, after a meal. If the fly over-fed itself, it might not be able to discharge the last bolus of blood from the sucking stomach.

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[1] Gray and Greig (**18**) report trypanosomes in the "ventral food reservoir" (sucking stomach?) twelve hours after feeding. Stuhlmann (**41**) prefers the name "crop" for the sucking stomach.

When the digestive tract is gorged with blood it is so distended, and the wall is so thin, that it is a difficult matter to dissect it out without rupturing it and letting blood escape. My colleagues, Gray and Tulloch, taught me a simple and effective method for doing this operation, which is as follows: The terminal segment of the abdomen is snipped off, the body is laid flat on an ordinary slide, and then a mounted needle, seeker, or other suitable instrument, is pressed down flat on the waist or base of the abdomen and passed along with an even, steady pressure towards the tip of the abdomen, so as to squeeze all the contents of the abdomen out on to the slide. With a little care and practice all the abdominal organs can be squeezed out quite uninjured, and can be separated from one another on the slide afterwards. In most cases the thoracic intestine and proventriculus are pulled out together with the abdominal contents. This simple method is most useful for rapid examination of a number of flies.

In order to make smears of the stomach-blood, it is necessary, as already stated, to mash it up with a little salt-citrate solution; for the intestinal and proctodæal contents this is not necessary. It is best to avoid, as much as possible, the use of salt-solutions. By comparing preparations made from pure blood with those of blood that had been mixed with citrate solution, I found the trypanosomes distinctly altered in form in the latter. Our method was to draw up the intestinal contents into capillary glass tubes, from which fine drops were expelled by blowing them gently on to slides, in order to make the smears. When made, the smears were sometimes dried rapidly in the air and then fixed with absolute alcohol or methyl alcohol, sometimes fixed with osmic vapour, employed in the manner recommended to me by my friend Dr. Plimmer: twenty drops of 4 per cent. osmic acid, with one drop of glacial acetic, placed in a stoppered tube of sufficient calibre to hold a slide, and the wet smear placed into the tube for about half a minute. I also tried a modification of this, as follows: A drop of blood on a slide was exposed, as rapidly as possible, to the osmic-acetic vapour for about half a minute, then mixed with an equal-sized drop of equal parts of fresh serum and dilute glycerine, and the whole smeared out. Subsequently the smear was fixed with absolute alcohol, without letting it dry; the glycerine was used in order to keep it moist for any length of time. I found, however, that this method tended to shrink the trypanosomes, though successful in other ways. It would be necessary to experiment in order to find the exact proportion of glycerine that should be mixed with the serum. In general I found the form of the body much more perfectly preserved in osmic-fixed smears than in air-dried preparations; the trypanosomes appear solid and plastic in the former, and always more or less flattened out in the latter. It seems, indeed, inevitable that the violent method of drying must deform a soft protoplasmic body.[1]

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[1] "Der Rüssel wird . . . während des Saugens häufig sägend auf- und abbewegt," Stuhlmann (**41**, p. 4). My statements in the text were written before Stuhlmann's memoir came to hand.

I wasted, I am sorry to say, a great deal of precious time trying to stain trypanosomes by the ordinary technique, which gives such good results with other Protozoa, especially the various carmine and logwood stains, which all proved useless. It is a puzzle to me why the Protozoa parasitic in blood should be so entirely different from other Protozoa in their staining reactions. I have always experienced just the same difficulty in trying to stain other Protozoa by the methods so successful for blood parasites. I fell back, finally, entirely upon the classic Romanowsky stain, using either Leishman's method, Laveran's Bleu-Borrel method, or Giemsa's stain. Latterly I used Giemsa's stain entirely, differentiating with tannin-orange solution (Unna's, obtained from Grübler). As a rule the smears were "refreshed" with fresh blood-serum, after the procedure recommended by Leishman. I kept my smears uncovered. Those that have been much used for study are now, I find, deteriorating, but those that have not been much looked at seem to be quite unaltered. Frequent baths of cedarwood oil and xylol alternately effect the stain after a time. Some smears, owing to pressure of time, were left unfixed, and were fixed after my return; none of these were very good.

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[1] Lühe (26, p. 70), makes some valuable remarks on this point. Compare also Plimmer (35).

Parasites of Glossina palpalis.—I examined the contents of the digestive tract and other organs in freshly-caught hungry flies with the object of making myself acquainted with the native parasites, if any, of the fly, in order to avoid confusing with them the stages of trypanosomes. Except for bacteria, however, the fly was very free from internal parasites. No gregarines or other sporozoa were found; it would, indeed, be improbable that a tsetse-fly, which apparently feeds exclusively on the blood of vertebrates, should acquire an infection of such parasites, which, as a general rule, are taken up with food in an encysted form by their hosts.

The commonest object in the gut was a large bacillus (figs. 111–123), apparently always present, sometimes in enormous numbers, especially in the stomach. When plentiful they occurred in masses or bundles; in such cases the flies always appeared sickly, emaciated, and anæmic, and usually had the digestive tract empty of blood, even when they had been put on to an animal; whether through the fly refusing to feed, or through rapid absorption of the blood by the bacteria, was doubtful. These bacteria show, apparently, only Brownian movement.[1]

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[1] Stuhlmann (**41**, pp. 38, 39), has seen these or similar bodies in G. fusca. He believes them to be protozoa and not bacteria, and considers that they are not parasites because they are found to be invariably present. I cannot agree with him in either of these conclusions.

In the proctodæum and hinder part of the intestine I noted "sausage-shaped protoplasmic bodies, apparently flagellate, swimming actively" (figs. 124, 125). These were perhaps a form of the bacterium already described, as I noted others that appeared to be intermediate between the two forms.

In the same regions I noted also in one fly slender thread-like organisms, wriggling actively; perhaps spirochætes. I never found them, however, in my smears. In one fly (batch of Oct. 1st, 1905, examined Oct. 5th) I noted the red blood in the stomach "swarming with small motile bacteria, not the large inert forms ordinarily found." This fly was one bred out in the laboratory.

I frequently found, in the blood taken from tsetse-flies, and in my smears, curious bodies apparently of vegetable nature; figs. 127–131 show rough sketches from life of these bodies; fig. 126 is from a smear, drawn with the camera lucida, magnified 2000 linear. As may be seen, they are more or less spindle-shaped bodies, sometimes enclosed by a distinct membrane, sometimes not; the contents are divided into two, four, or more cells, containing each one or two nuclei. The nuclei sometimes appear in the living condition as clearer spaces, sometimes as darker spots. The colour of these bodies in life is greyish with a slightly greenish or brownish tinge. My notion of these bodies was that they came really from the solutions of salt or sodium citrate used in the dissections. Quite recently examining the blood of a rudd (Leuciscus erythrophthalmus) for trypanosomes, in Norfolk, I was surprised to find a quite typical example of these organisms. In this case the blood, in which no trypanosomes could be found, had been taken from the heart of a fish by means of a capillary glass tube, which had been previously washed through with normal saline solution. Hence there was a possibility that the body seen might have come from the salt solution. It may, however, be some form of organism inhabiting blood. I content myself with noting the occurrence of these bodies fairly frequently in tsetse-fly preparations.

In one fly I noted oval, refringent, motionless bodies in the proctodæum. In the sucking stomach I noted, in the wall, "long, greenish-yellow filaments, consisting each of a row of joints of varying lengths."[1]

Nomenclature of the structural parts of trypanosomes.—There is much diversity in the names applied by different authors to some parts of the trypanosome body, more especially with regard to the smaller chromatic nucleus, and also in the use of the terms anterior and posterior. The smaller nucleus is termed usually in England the micronucleus; in France the centrosome; and in Germany the blepharoplast. Each of these terms is open to objection, the term micronucleus, in my opinion, least of all, so long as it is used in a purely descriptive sense to mean simply a small nucleus; the danger is, however, that it leads to instituting a comparison with the micronucleus of Ciliata, which is a totally different structure, a reserve generative nucleus. Whatever the nature of the micronucleus of a trypanosome may be, there is no evidence that it is composed solely of generative chromatin.

The terms blepharoplast and centrosome raise the whole question of the nature of the bodies so named—a question upon which it is possible to have much difference of opinion. It is enough for me to refer here to the recent memoir by Goldschmidt and Popoff (**16**), in which the subject is discussed at length, and to state my own views: I regard a centrosome as an achromatic body, in connection with a nucleus; and a blepharoplast as a body of the same nature as a centrosome, but in connection with a protoplasmic locomotor apparatus, such as a flagellum or cilium. This is the sense in which I understand these terms; others may differ from me.

With regard to trypanosomes, I may point out, in the first place, that the "micronucleus" is certainly a chromatic body and cannot be classed with achromatic structures. Apart from its staining reactions, which are more intense than those of the larger nucleus, I may cite the observations of Schaudinn (**40**), who describes it as arising in Trypanosoma noctuæ by an unequal division of the zygote nucleus; and I may further draw attention to the condition in Trypanoplasma, where it is as large as, or even larger than, the other nucleus, so that the term micronucleus for it becomes rather a misnomer.

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[1] Compare Stuhlmann (**41**, p. 47).

In the second place, I may point out, as others have done also, that the flagellum does not arise from the smaller nucleus, but quite independently of it, from a minute basal granule.[1] The behaviour of the flagellum and of the smaller nucleus in division also shows clearly their complete structural independence, as I have pointed out below (p. 193).

I consider the basal granule of the flagellum as a true blepharoplast in the sense in which I have defined the term above; and I regard the nuclear apparatus of trypanosomes as specialised into two distinct portions, one regulating the function of locomotion, the other that of nutrition. Hence I consider that the terms kinetonucleus and trophonucleus, suggested by Woodcock (**42**), express most correctly the true nature of these bodies, and I shall employ these terms in my descriptions, though it is often convenient to speak of the trophonucleus simply as the nucleus, and as a further abbreviation I shall sometimes refer to the two bodies as n and N simply.

From the true blepharoplast arises the flagellum, which passes to the surface of the body and runs along the edge of the undulating membrane as the marginal flagellum, until it reaches the end of the body, where it becomes a free flagellum of greater or shorter length. According to Schaudinn's observations on the formation of the locomotion apparatus in Trypanosoma noctuæ (**40**), there exists also a distal blepharoplast, as it may be termed, situated at the free end of the flagellum. It is stated that the flagellum and undulating membrane are formed from the achromatic apparatus of a nucleus spindle, of which the central spindle gives rise to the flagellum and the mantle fibres to the myoneme-fibres of the undulating membrane, while the two centrosomes become the blepharoplast. These statements have received some confirmation from the observations of Robertson (**37**) on the formation of the flagellum in the trypanosome of Pontobdella muricata.

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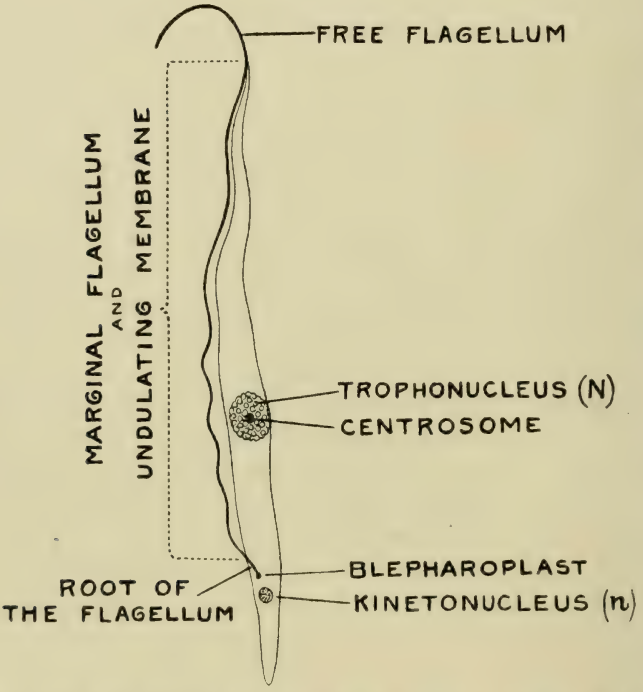
[1] Dutton, Todd, and Hannington (**15**, p. 219) point out that "the thickened end of the undulating membrane ends not in it [*n*] but in a pinkish basal granule or 'diplosome'" (why "diplosome"? It is only double when about to divide). Compare Novy (**33**), pp. 5 and 6.

I have frequently noticed, especially in the slender forms of T. grayi, that the flagellum is distinctly thickened at the free extremity, but I should not like to affirm the existence of a definite blepharoplast at this point. Prowazek (**36**) has also described for T. lewisi a complicated arrangement of anchoring granules and fibrils. I can only say that in my material I have not seen them.

With regard to the use of the terms anterior and posterior much has been written, and it has been pointed out by Woodcock (**42**) and Lühe (**26**) that what is morphologically anterior in one trypanosome may be posterior in another. It cannot, I think, be disputed that there may be two entirely different lines of phylogenetic evolution amongst the organisms grouped generally as trypanosomes (see below, p. 219), but in the present state of knowledge it is not possible to state definitely which of the two possible modes of orientation is applicable to a given species. Hence it would be best, perhaps, to avoid altogether the use of the terms anterior and posterior in describing trypanosomes, and to speak of the flagellar and anti-flagellar extremities, but such a terminology becomes very cumbrous in practice. I shall speak of the flagellar extremity as anterior, the anti-flagellar as posterior, using the terms in a purely descriptive sense and without prejudice to the morphological questions involved. So far as I have observed, when trypanosomes are moving freely they travel usually with the flagellar extremity directed forwards, but when pushing their way amongst blood-corpuscles they do so with the free flagellum of the undulating membrane directed backwards, their movement in the latter case being similar to that of a Trypanoplasma.

The accompanying diagram (Text-fig. A) is intended to make clear the terms and the use which I shall make of them in this memoir.[1]

Text-fig. A.



II. Observations on Trypanosoma gambiense.

(A) T. gambiense in the Blood of the Vertebrate Host.

My observations relate chiefly to T. gambiense in the blood of monkeys, all of which had been infected, directly or indirectly, by inoculation with cerebro-spinal fluid from sleeping sickness patients, with the exception of one, which became infected by the bites of tsetse-flies caught in the neighbourhood of Entebbe; I shall refer to this monkey briefly as the fresh-fly monkey. I have also preparations of cerebro-spinal fluid from patients, and of the blood of a chimpanzee, eighteen days after inoculation with human cerebro-spinal fluid. I have no preparations of human blood showing trypanosomes.

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[1] See also Appendix (p. 252).

In all cases alike the trypanosomes are distinguishable by their general build and appearance into slender, stout, and intermediate forms.[1] In the living condition the slender forms appear more snake-like, and are active in their movements, while the stout forms are fish-like in shape, and less motile. In successful preparations of blood-smears the three forms can usually be distinguished readily, especially in films fixed with osmic acid vapour, which preserves the body-form very perfectly. In films preserved by the ordinary drying method the different forms appear less differentiated. This is evidently the result of a slight flattening-out of the body, produced by the method of drying. The two extremes of the series—namely, the stout and slender types—contrast with each other, not only in their proportions, but also in the relative length of the free flagellum, which is long in the slender forms, short in the stout ones. The trypanosomes of the intermediate type can also be subdivided into two types by this character, so that all the trypanosomes in a given blood-preparation can be divided into two classes—those with long and those with short flagellum. This character, which is plainly seen in the figures of Bruce and Nabarro (**5**), is more reliable for use as a morphological distinction than the stoutness of the body—a character liable to alteration or deformation due to imperfect preservation.

The extreme of the stout type is seen in the so-called

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[1] Moore and Breinl (**30**) state that they are unable to distinguish any marked dimorphism, and that the so-called males and females are "arbitrarily chosen" extremes in a continuous series. It was never pretended that they were anything else than the extreme differentiations, obvious naturally, of a neutral or intermediate type; as will be described below, after twenty-four hours in the invertebrate host the intermediate forms disappear, and only the extremes remain, arbitrarily selected or differentiated by the action of their environment.

"stumpy" forms (figs. 9, 10, 17, 20, 21, 33–35), in which the body is short and stout, usually rounded off abruptly at the posterior end, and with a very short flagellum. These stumpy forms are not always to be found, but when present are unmistakable. I found the greatest contrast in the trypanosomes from cerebro-spinal fluid (figs. 30–35), in which the slender and the stumpy forms recall the two types of Trypanosoma dimorphon, and very few intermediate forms were to be found. A count made from four smears of cerebro-spinal fluid gave twenty long and slender forms (43·47 per cent.), eighteen short and stumpy forms (39·12 per cent.), of which one was dividing, and eight intermediate forms (17·39 per cent.). In two preparations, made from the blood of the same monkey (478) on two successive days (pp. 230, 231), I find on one day (figs. 1–5) no stumpy forms, though they were present in smears made the next day (figs. 6–10); and it is noteworthy that in flies fed on this monkey on the first day I obtained no development of the trypanosomes, while the batch of flies fed two days later showed a normal type of infection (p. 231). My best infection of T. gambiense in the tsetse-fly was from a batch of flies fed on monkey 478 on a day (Oct. 19th, p. 237) when it was showing trypanosomes, few in number, but sharply differentiated in form (figs. 11–13).

Castellani (**13**) has stated that T. gambiense, i. e. the trypanosome of Gambia fever, moves with the flagellum forwards, that of the sleeping sickness with the blunt end forwards. I find that the trypanosomes I have observed push their way through blood-corpuscles with the blunt end forwards, but when moving freely they tend to travel with the flagellum forwards.

With due allowance for variations in form and size, I find the structure of T. gambiense very uniform. The nucleus is large and lodged near the middle of the body; it appears to be a compact mass of chromatic granules, without any definite limiting membrane. Near the nucleus a few coarse granulations are commonly seen, which may, however, be few in number or absent. The kinetonucleus is usually close to the posterior end, but may be a short distance from it when the posterior end is prolonged into a point. The kinetonucleus is usually a minute round body, but may be rod-shaped, a form possibly connected with impending division, though this seems to me doubtful. I observed that in slender forms the kinetonucleus, if rod-shaped, was placed transversely (figs. 12, 30), but in stumpy forms the rod-shaped kinetonucleus was applied to the surface of the body and had a more longitudinal direction (figs. 34, 35). In good preparations it can always be seen clearly that the flagellum does not arise directly from the kinetonucleus, but from a minute granule, which, as stated above, I regard as the true blepharoplast. In some cases the blepharoplast may be very close to the kinetonucleus, or may be over or under it in the preparation, giving the impression that the flagellum arises directly from the kinetonucleus. The marginal flagellum stands well off from the body, forming an undulating membrane of some depth when seen in profile. The distinction between body and undulating membrane is sharp in osmic preparations, but less so in films fixed by drying, owing, I believe, to the deformation and flattening out of the body produced by the latter method. The flagellum takes, in fixed preparations, a variable number of turns, corresponding to pleats in the undulating membrane, which depend probably on the movements of the animal at the time when it was fixed; but as a rule the membrane is least pleated in the extreme forms, both slender and stumpy (compare figs. 5 and 13), most so in the intermediate form. The anterior end of the body is prolonged into a slender filament, running parallel to the flagellum, sometimes nearly to the end of it.

Most previous observers have described and figured T. gambiense as being vacuolated (see especially Bruce and Nabarro [**5**] and Castellani [**13**]). It has been regarded as almost a normal feature of the species to have a vacuole near the kinetonucleus.[1] In my experience these vacuolated forms are very rare. I have found them only in trypanosomes from the cerebro-spinal fluid, never in those from the blood, and in the former they are by no means universal. I consider the vacuolated condition as an abnormal one, due to the parasite being in unfavourable conditions, or to the reaction upon it of a diseased enfeebled host. Bruce, Nabarro, and Greig (**6**, p. 20) express the view that trypanosomes do not find cerebrospinal fluid so favourable for growth as blood, and are stunted; they also note (loc. cit., p. 32) post-mortem forms, with large vacuoles, and deformed. Plimmer (**35**) regards the vacuolated form as "probably due to some condition of environment."

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[1] "Für besonders charakteristisch wird von einiger Forschern eine Vakuole gehalten, die . . . . nicht immer in gleicher Deutlichkeit hervortritt," Lühe (**26**), p. 115.

Prowazek (**36**) has given three figures of T. gambiense (named by him T. castellanii), all of which differ a good deal from the appearances I am accustomed to see. His first figure (loc. cit., fig. 108) looks like a stumpy form with a vacuole, but it has a long free flagellum, and is perhaps an ordinary form deformed. His second figure (loc. cit., fig. 109) shows a long slender form, evidently in process of division, as *n* and *N* are divided; that being so, one looks at once for the split flagellum which would naturally be found at this stage. The author has figured in the cytoplasm a slender filament which I identify, without hesitation, as the daughter flagellum not traced for its whole length. I think this figure throws some light on the fibrils Prowazek introduces into so many of his figures of trypanosomes. The author's third figure shows a trypanosome "with long, narrow nucleus." Never in all the many trypanosomes of this species that have come under my observation have I seen one with a nucleus such as Prowazek figures in this specimen; I am inclined to regard it as one in which the nucleus has become deformed in the process of smearing.

In short, I can but state that Prowazek's figures represent forms which, in my experience, are aberrant and abnormal; whether this is to be explained by the influence of technique on the parasites, or of a European climate on their hosts, I leave an open question. I am convinced, however, that the appearance, and even the structure, of trypanosomes may be greatly affected by the condition of their hosts.

I am also unable to make head or tail of the "atypical" forms described by Castellani (**13**). I have never seen anything like them. I note that he states that they occur especially in the last stages of disease. As my object was to trace the normal life-history of the parasite, I avoided as much as possible all material in which the parasites were likely to be influenced by a sickly condition of the host. Perhaps that accounts for the great discrepancy between my observations and those of these investigators with regard to the vacuolation of the trypanosome.

(B) The Development of Trypanosoma gambiense in Glossina palpalis and other Diptera.

My observations and experiments on the fate of T. gambiense in invertebrate hosts were carried out chiefly on Glossina palpalis, but I also made a few observations on the species of Stomoxys, common at Entebbe, and on the two common Entebbe mosquitoes, one a species of Tæniorhynchus, the other of Mansonia.

The results obtained with Glossina palpalis were remarkably uniform, and will be briefly summarised before entering into full detail. The trypanosomes at first multiply in the digestive tract of the fly, and by twenty-four hours are found to be differentiated into two types, slender and stout, sharply distinct from one another, with no intermediate forms. The next day, that is, at about forty-eight hours, these two types are succeeded by a more uniform type, so far as structure is concerned, but varying from slender to fairly stout, with all possible transitions, and of considerable length. On the third day after infection trypanosomes are always to be found in the digestive tract, and are forms of great length, relatively, varying from slender to stout, sometimes appearing degenerate in structure and diminished in number, but in other cases numerous, active, and with no signs of degeneration. On the fourth day trypanosomes are very rarely to be found, and, if present, are very scanty in number and of large size, but are usually absent altogether. In no case have I found any signs of T. gambiense in the tsetse-fly later than the fourth day after infection. Nabarro and Greig (**31**) found T. gambiense in G. palpalis up to seventy-one hours after feeding; animal trypanosomes up to 100 hours. Throughout these four days of development T. gambiense undergoes a steady and well-marked increase of size.

In the other Diptera I found that T. gambiense went through the same changes of form and structure as in Glossina palpalis. In Stomoxys, however, no trypanosomes were found on the second day (forty-eight hours) after infection. My mosquito experiments are very incomplete, but I found active trypanosomes in Tæniorhynchus as late as seventy-two hours after infection.

In spite of much searching T. gambiense was never found in any organs except those in which digestion of the blood was proceeding; that is to say in the stomach and intestine.

I will now proceed to a more detailed description of my observations.

Preparations made from flies shortly after infection (July 31st, p. 228, and Sept. 8th, p. 229) show the gradual disappearance of the trypanosomes of intermediate type by their conversion partly into slender forms, but chiefly into stout forms. I think it is safe to assume that the intermediate forms with long flagellum become converted into slender forms (compare figs. 41–44), while those with short flagellum become stout forms. There are also many dividing forms found at this period, indicating active multiplication. The process of differentiation is complete twelve hours after infection, and then we have the two sharply marked types, slender and stout, which characterise the first day after infection. Seen in the living condition the slender, twenty-four-hour forms appear transparent, of serpentine appearance, and very active; the stout forms are fish-like or whale-like in form, opaque and granular, and sluggish in movement (figs. 102, 103).

The slender forms (figs. 45, 48, 52, 56–58, 61, 62, and P. R. S., B 78, Pl. XII, figs. 1–6) are distinguished in preparations, not only by their snake-like form, but also by the clearness of their cytoplasm, which is free from granulations, and remains scarcely, or not at all, tinged by the Romanowsky method of staining. Comparison of earlier stages show that these slender forms arise from the slender or intermediate forms met with in the blood before it is taken up by the fly, by absorption of the granules in the cytoplasm. The whole appearance of these forms suggests the activity and mobility which they are seen to possess when observed in the living state. The flagellum is very long and stands well off from the body, but the undulating membrane is not greatly pleated. The kinetonucleus is a circular dot usually placed at or very near the posterior end, but sometimes at a short distance from it. The nucleus has a dense, compact appearance, and usually a compressed form, but very frequently an irregular outline, with sometimes an appearance as if portions of chromatin were being detached from it, as noted in my preliminary communication (fig. 56, and P. R. S., B 78, Pl. XII, figs. 4, 5, and 6).

The stout forms (figs. 46, 47, 49–51, 53–55, 60, 63–65, 76, and P. R. S., B 78, Pl. XII, figs. 7–14), on the other hand, are large and obese, with cytoplasm coarsely granular and staining deeply. The flagellum is short, the undulating membrane but slightly pleated, and the kinetonucleus, which is circular or rod-shaped, is usually some distance from the posterior end, a point in which they contrast with the "stumpy" forms found in the vertebrate body. Sometimes the posterior end is prolonged into a short "rostrum," a feature which becomes exaggerated at a later stage. The nucleus is large and loose in texture, but of definite outline, often with a peripheral ring of coarser granules, but never showing the appearance of chromatin being ejected, as in the slender forms.

Not only do these two forms of trypanosomes differ in structure and appearance, they also show a curious difference in the mode of division. When the slender forms divide the two daughter-kinetonuclei keep close together at the posterior end (fig. 217; see also P. R. S., B 78, Pl. XII, fig. 6); but in the stout forms, when the division has reached a certain stage, one of the two daughter-kinetonuclei passes forwards and takes up a position between the two daughter-nuclei, thus producing a characteristic division-stage (compare figs. 54, 55, and P. R. S., B 78, Pl. XII, fig. 12).

On the second day, that is, about forty-eight hours after infection, the trypanosomes are seen to be changing into a type which reaches its perfection on the third day, and is best described from its later development. What I will call the third-day type of trypanosome is of considerable length, appearing under forms both slender and stout, but with transitions between these two variations (figs. 83–96). The body is generally cylindrical, tapering gradually anteriorly and bluntly rounded off posteriorly. The kinetonucleus is round or rod-shaped, sometimes large, and generally situated some distance from the posterior end. The undulating membrane is not much pleated, and the free flagellum is short, even in the more slender forms (fig. 86).

In the preparations of about forty-eight hours we find this type sometimes fully perfected, sometimes only beginning to make its appearance. In one fly I found in the red blood forms similar to those described above as characteristic of twenty-four hours after infection (figs. 76, 77), while the black blood showed forms more advanced towards the third-day type (figs. 73–75). The question at once arises, how does this change of type come about? On account of the uniformity of structure shown by the third-day type I am inclined to derive them all from the stouter type seen at twenty-four hours, and to regard the more slender forms seen on the third day as derived by divisions from the stouter forms. In that case what becomes of the remarkable slender forms seen at twenty-four hours? It would be a tempting hypothesis to suppose that they have conjugated with the stout forms, and that the big forms of forty-eight and seventy-two hours represent zygotes, but I am unable to bring forward any facts in support of this supposition. If the stout twenty-four-hour forms give rise to all those found later, it is implied that the slender twenty-four-hour forms die off; it is, of course, possible that they may persist and give rise to the later slender forms, and I must confess that my observations do not enable me to decide this point with certainty.

In my smears of flies dissected on the third day, I find great differences in the condition of the trypanosomes. In those from one batch (Oct. 18th, p. 235) the trypanosomes were almost without exception excessively granular and frequently also very vacuolated. They gave me the impression of being degenerate forms, with impaired vitality (figs. 82*a*–88). In another batch (Oct. 22nd, p. 237), however, I found in my smears abundant healthy-looking trypanosomes (figs. 89–92), not vacuolated nor excessively granular, and in the living condition they were extremely active, so much so that I thought I had to do with T. grayi.

In both cases alike, however, no trypanosomes were to be found in the flies dissected the day following.

On the fourth day (ninety-six hours or so) I have very rarely found trypanosomes present in the fly, and only once in smears of this period (Sept. 12th, p. 230). My preparations of them are, unfortunately, very poor, but it is at least possible to trace the form and features of the trypanosomes, which are of a large type, differing in no essential particular from those of the day before (figs. 97–101).

In no case did I ever find T. gambiense in the fly after the fourth day.

The disappearance of T. gambiense from the gut of the tsetse-fly, on or after the fourth day after infection, may mean either that the trypanosomes die out completely, or that they pass into some form which has not been recognised. If they really die out in the fly without completing any life-cycle, it would indicate, in my opinion, that Glossina palpalis is not the true host for this trypanosome, and that some other invertebrate host must be sought for it. I discuss this question more fully below. If, however, the disappearance of the trypanosomes is only apparent, and they really persist, there are many possibilities which suggest themselves. First, they might pass into some other organs of the fly; against this I may urge that I have repeatedly examined all organs of the fly likely to harbour trypanosomes at various periods after infection, and never found a trypanosome or anything that suggested a stage of a trypanosome, outside the digestive tract. Secondly, they might assume some minute ultra-microscopic form; in that case, however, the function in the life-cycle of such a form would almost certainly be that of infecting a new vertebrate host by inoculation, and every experiment to produce infection with Glossina palpalis more than forty-eight hours after the fly had infected itself gave negative results. A third possibility is suggested to me by my observations on the encystation of T. grayi; it is possible that the trypanosomes which disappeared from the stomach and intestine passed on into the proctodæum in order to become encysted there. It is a matter of the deepest regret to me that I did not make smears of the proctodæum, but the idea of such a possible development of the trypanosomes was not present in my mind when I was in Entebbe; since I never saw T. gambiense in the proctodæum, I did not make any preparations of this region, and so the golden opportunity of deciding this point was lost to me.

Dutton, Todd, and Hannington (**15**) have published observations on the fate of T. gambiense after being taken up by various Arthropods. In G. palpalis they find "unaltered parasites in the alimentary canal up to forty-eight hours; living but altered trypanosomes up to seventy-two hours after feeding." This does not agree with my experience; I find the trypanosomes beginning to alter in character a few hours after feeding; by alteration, however, the authors mean, apparently, coarse or violent modification of trypanosome-structure. In Stomoxys trypanosomes were found up to twenty hours after feeding. In the larva of Auchmeromyia luteola they were found up to twelve hours after feeding; in Anopheles up to forty-two hours. Trypanosomes were also obtained in a louse. There is nothing that calls for notice in their observations except the "rounded forms," which they believe to "arise from the englobation of single trypanosomes which have cast off blepharoplast and undulating membrane and become spherical." The authors describe their formation more in detail in the rat flea (louse ?), in which they note "granular, indistinct, obviously degenerating parasites." I regard the rounded form seen by Dutton, Todd, and Hannington as trypanosomes succumbing to, and being digested by, the digestive juices of the alimentary canal, and quite distinct from the rounded forms described by Koch (**20**) and Stuhlmann (**41**), which probably form part of a true developmental cycle. I have not found rounded forms of T. gambiense in any of my preparations.

III. OBSERVATIONS UPON TRYPANOSOMA GRAYI.

My material of this trypanosome is limited, because, for reasons explained above, I did not systematically search for it in Glossina palpalis, so that all the instances of its occurrence that came under my ken were in flies used by me for studying the changes undergone by T. gambiense. In this way I found in all six flies containing T. grayi. Fortunately this trypanosome, when it occurs at all, is found in such vast swarms that a single infected fly furnishes an abundant material. Hence, I have been able to discover some facts of interest relating to this form, notably the process of encystation, not previously observed, so far as I am aware, in any trypanosome, and I have also studied carefully the distribution and occurrence of the many forms of this trypanosome in the different regions of the intestine of the fly; but with regard to this latter point, it is to be regretted that my material is defective, inasmuch as none of my five flies had any trypanosomes in the proventriculus, so that I have had no opportunity of studying the forms occurring in this part of the digestive tract. In spite of much searching I have never found this trypanosome in any organs of the fly other than the digestive tract (stomach, intestine, and proctodæum). Of the six flies infected by T. grayi that I studied, in one case (Nov. 2nd, p. 238) I did not make any notes, unfortunately, as to the exact provenance of the trypanosomes. With regard to the other five, one (Nov. 10th, 1st fly, p. 241) had the trypanosomes only in the proctodæum. A second fly (Nov. 10th, 2nd fly, p. 241) had evidently not fed recently, and contained only a small quantity of black blood in the intestine, and trypanosomes were found only in the black blood and the proctodæum. The remaining three flies (Nov. 13th, p. 243, Nov. 14th, p. 244, and Oct. 10th, p. 232) showed trypanosomes swarming through the red blood (stomach), black blood (intestine), and proctodæum. In my preliminary account of the encystation (P. R. S., B 79, p. 35) I stated that it was rare to find them in the proctodæum, but I now recognise that this was a mistaken impression on my part; indeed, my limited experience indicates rather that the trypanosomes of this species always occur in the proctodæum, even when they are absent in other parts. But I have never found T. gambiense in the proctodæum.

While I was at Entebbe I had little time to draw and study accurately my preparations, but I made a few drawings of my slides from one fly (Oct. 10th), and some of them were published in the preliminary report by Gray, Tulloch, and myself (P. R. S., B 78, Pl. XIII, figs. 23 and 28). I have since then examined my slides of this fly much more carefully, and found that in this case also the proctodæum contained vast swarms of the trypanosome (p. 233), a fact which I had overlooked at the time of writing my report.

Trypanosoma grayi occurs under such a bewildering variety of forms and sizes that it is a matter of great difficulty at first to see any order or regularity in them. This difficulty is increased by the fact that in one fly the trypanosomes will be found reaching a much larger size, as a whole, than in another; thus, in the flies of Nov. 10th (2), Nov. 14th, and Oct. 10th, some of the trypanosomes are of very large proportions, while in the fly of Nov. 13th they are much smaller and more slender in build. From my observations I have come to the conclusion that all the different forms may be grouped in three classes, the first of which has many subordinate subdivisions. I distinguish the three classes recognised by me in the following manner: (1) Ordinary or multiplicative forms; (2) slender forms; (3) Herpetomonas forms.

(1) The ordinary forms occur in a variety of sizes due principally to their growth and multiplication. We may distinguish, in the first place, adult or full-grown forms—those of the largest size. These are elongated forms, with the body more or less cylindrical in build; in some the body tapers anteriorly to a fine point (figs. 140, 158, 173, 218, etc.); in others, on the contrary, the body diminishes in thickness very gradually towards the anterior end, up to a point at a short distance from the extremity, from which it narrows rapidly to a stumpy point (figs. 138, 139, etc.). We may conveniently distinguish these two forms as the serpentine and the vermiform respectively; the free flagellum is short in both, but appears longer in the serpentine form, perhaps, in some cases, on account of the difficulty in distinguishing the exact anterior termination of the body. A third variety of the large forms is the tadpole form, in which the posterior end of the body is swollen out (figs. 142, 221, etc., and P. R. S., B 78, Pl. XIII, figs. 23, 26); these are the forms which I formally identified as females, but I am now inclined to regard them simply as full-grown forms which are about to multiply by division.

The process of division, which will shortly be described in detail, leads to a small daughter-individual being split off from the larger parent-individual; in this way young forms are produced (fig. 220, etc.)—the smallest individuals of the ordinary type. These young forms are always present, and frequently very abundant (Nov. 14th, p. 244). Between them and the largest forms every gradation of size is to be found; hence we may safely assume that the small daughter-forms produced by division grow up in time into the large forms, unless they develop into some other type, as will be described presently.

To sum up, we may classify the ordinary type of T. grayi roughly into serpentine, vermiform, tadpole-like, young, and intermediate (i. e. growing) forms. To these, however, must be added a sixth form, the significance of which is not at all clear to me. These are the round forms frequently present, and obviously connected with the young forms. It is easy, when round forms occur, to trace a series of transitional forms between them and the young forms, but whether round forms become young forms, or vice-versâ, it is not so easy to determine. A typical round form has a more or less spherical body, giving off a flagellum, which is entirely free except at its origin (fig. 136). Gray has figured a further development of the round forms, in which the flagellum becomes wrapped round the body in such a way as to simulate a cyst or cuticle in optical section (P. R. S., B 78, Pl. XIV, figs. 44–48), and also forms in which the flagellum appears to be entirely lost (loc. cit., figs. 49–51). I have not found such forms in my preparations, but I have seen those that my colleague has figured. According to Koch (**19**) and Stuhlmann (**41**), such forms are a regular part of the life-cycle, and Robertson (**37**) also describes them in the trypanosome of Pontobdella. Koch describes them as arising by the break-up of large, multinuclear forms, possibly zygotes. Robertson, on the other hand, considers them to be the first form assumed by trypanosomes when taken up from the blood of the vertebrate. Both Koch and Robertson describe the formation of small forms from the round forms.

As round forms are not very frequent in my preparations, I am not able to make any definite statements about them, except that they are connected by transitions with small forms. I am inclined to regard them as a normal temporary resting phase of the small forms, though in a few cases, perhaps, they may be due to imperfect fixation or other deformation due to technique—for instance, alterations during the dissection of the fly before fixation, when owing to any cause the making of smears has been delayed.

In all the different varieties of the ordinary form, as I have called it, of T. grayi, the structure of the body is in general very uniform, though showing great variations in some points. As regards the cytoplasm, I find remarkable differences, which are evidently due to differences in the action of the stain used (Giemsa's mixture); two types of staining are produced which are well exemplified in the series of dividing forms figured by me (figs. 204–216), all of which are drawn from two preparations made from smears of the same blood, and stained in Giemsa's solution side by side in the same dish. In one preparation the cytoplasm is bluish in colour and shows coarse granulations deeply stained, while the flagella are very faintly stained, appearing sharp and delicate, often difficult to make out clearly (figs. 204, 207, 212, 213, 215, 216). In the other preparation the cytoplasm is reddish in tint and the granulations are scarcely seen, or not at all, while the flagella are deeply stained and appear thick and very distinct (figs. 205, 206, 208–211, 214). The differences in the staining are probably due to differences in the use of the Tannin solution, used to differentiate the stain (see p. 169).

The nucleus (*N*) of T. grayi presents itself as a clump of granules, sometimes compact, oval or round, and in the latter case sometimes showing a distinct rosette-like structure (fig. 182, and P. R. S., B 78, Pl. XIII, figs. 35, 39); more often the nucleus is quite irregular in shape and loose in texture, and in one fly (Nov. 10th, [2], p. 241) the nucleus shows streaks of granules apparently trailing irregularly out from it, in nearly all the large forms (figs. 138–140). This suggests that the coarse granulations of the cytoplasm represent chromidia derived from the nucleus; but they stain slightly differently with Giemsa's stain, the granules of the nucleus having a redder tint, while the chromidial granules are more purple, like the kinetonucleus in tint.

The kinetonucleus (*n*) is large and rod-shaped, its length being about twice its breadth; it is superficial in position, and often appears to bulge from the surface of the body. In very thin smears it is often torn out of the body. It is very compact and stains deeply, so that no structure can be made out in it.

The most remarkable variations can be seen in the relative positions of *n* and *N*. I distinguish six types[1]: (1) *n* well in front of *N*, with distinct space between (figs. 181, 182); (2) *n* just in front of *N*, with no space between (fig. 156); (3) *n* is at the side of, or overlapping *N* (fig. 175); (4) *n* just behind *N* (fig. 176); (5) *n* far behind *N* (fig. 160); and (6) *n* terminal (fig. 152). Miss E. Y. Thomson has made some counts for me of the different types, which I record below:

		(1)	(2)	(3)	(4)	(5)	(6)
Nov. 13. Red blood	Numbers	1	30	45	47	320	7
(figs. 151–155, p. 243)	Percentage	0·22	6·66	10	10·4	71·11	1·5
Nov. 13. Black blood	Numbers	28	145	73	132	346	—
(figs. 156–164, p. 243)	Percentage	3·8	20	10	18·2	47·7	—
Nov. 13. Proctodæum	Numbers	11	55	33	62	179	5
(figs. 165–170, p. 244)	Percentage	3·1	15·9	9·56	17·9	51·5	1·4
Nov. 10. (2) Black blood	Numbers	113	332	168	65	17	—
(figs. 138–143, p. 241)	Percentage	16·3	47·9	24·1	9·3	2·4	—
Nov. 14. Red blood	Numbers	8	158	108	48	42	—
(figs. 171–173, p. 245)	Percentage	2·19	43·4	29·67	13·18	11·53	—
Nov. 14. Red-black blood	Numbers	40	209	863	314	207	—
(figs. 174–176, p. 245)	Percentage	2·4	12·8	52·8	19·2	12·6	—
Nov. 14. Black blood	Numbers	678	8	155	20	50	—
(figs. 177–182, p. 245)	Percentage	74·4	0·8	17	2·1	5·4	—
Oct. 10. Black blood	Numbers	14	56	49	14	6	—
(figs. 226*a*–231, p. 233)	Percentage	10	40·3	35·2	10	4·3	—
Oct. 10. Red blood	Numbers	25	44	39	3	3	—
(figs. 218–226, p. 232)	Percentage	21·9	38·5	34·2	2·6	2·6	—

These figures show in one fly (Nov. 13th) a great preponderance of forms with *n* posterior to *N*, or even terminal, especially in the red blood; but in all others the forms with *n* in front of or close beside *N* greatly preponderate. (In these statistics no account is taken of the slender and the Herpetomonas-like forms, in which *n* is invariably in front of *N*.) The forms with *n* posterior or terminal are interesting, as it is very probable that they represent very nearly the form under which T. grayi occurs in the vertebrate host; and in this connection their tendency to preponderate in the red blood is significant. I recognise now as erroneous my former statement (P. R. S., B 78, p. 243) to the effect that the forms with *n* posterior were nuper parentes, though this may be so in a few cases (see p. 192). It may be said, however, that for the forms occurring in the intestine of the fly, the normal and typical position of *n* is a little in front of *N*, for not only is this the condition found in those forms (the slender form and the Herpetomonas form presently to be described) in which the position does not vary, but it is also the position which *n* always assumes when division is about to commence.

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[1] A convenient graphic notation from these types is as follows:

(1) $\overset{n}{\underset{N}{=}}$; (2) $\overset{n}{N}$; (3) n/N; (4) $\frac{N}{n}$; (5) $\overset{N}{\underset{n}{=}}$; (6) $\overset{N}{\underset{n}{|}}$. A type with *n* terminal but anterior could be written $\overset{n}{\underset{N}{|}}$

I have made a detailed study of the process of division in T. grayi, with the object of being able to distinguish clearly between the stages of multiplication and of conjugation. The process of division shows some variations; I will first describe what appears to be the most usual course of events, and after that I will deal with the deviations from this course that are met with.

The first event in the process of division is that the minute basal granule or blepharoplast (see p. 172) of the flagellum divides into two (fig. 151). At the same time the kinetonucleus becomes slightly enlarged and elongated. Starting from the two blepharoplasts the flagellum splits longitudinally and as the two minute blepharoplasts are at first connected by a delicate thread, we obtain a common and characteristic early stage of division, in which, in a trypanosome otherwise of normal aspect, the flagellum shows the appearance of a loop at its proximal end (figs. 204, 205). The splitting of the flagellum continues and the two blepharoplasts travel away from each other, so that the appearance of a loop is lost; but at this stage there is nearly always seen a crossing over of the two halves of the flagellum in the region where it is split in two (figs. 206, 207). This crossing of the flagella marks a second characteristic stage during which the division of the kinetonucleus takes place. The splitting of the flagellum proceeds a certain distance and then breaks off, so that a longer and a shorter flagellum result from it (figs. 208–210). About the time the splitting of the flagellum is complete the nucleus divides. I have found stages of the division in which a thread or band ran between the two daughter-nuclei, connecting two granules, doubtless of centrosomic nature, imbedded each in the mass of one of the two daughter-nuclei, and difficult to distinguish from the chromatin granules surrounding them (figs. 209, 210). As may be seen from the figures, there is the utmost diversity in the relative positions of the two nuclei and the two kinetonuclei. I must therefore retract my former statement (P. R. S., B 78, p. 243) as to the constant position of n and N after division. When the division of the nucleus and flagellum is complete, the body begins to split (figs. 209–212), starting from the anterior extremity between the two flagella. The body always divides into two unequal portions, giving rise to individuals which are markedly unequal in size and may be distinguished conveniently as parent and daughter respectively. The parent takes the longer flagellum, that is to say, the principle portion of the original flagellum; the daughter takes the shorter flagellum. The two individuals are attached last in the neighbourhood of the kinetonucleus (fig. 212; compare fig. 217), until they finally break loose.

By some authors it has been stated that, in the division of trypanosomes, the daughter flagellum is not split off from the parent, but grows out independently of it. I have paid much attention to this point in T. grayi, and can find nothing to indicate that the new flagellum is formed otherwise than I have stated above, namely, by a process of splitting which starts from the division of the blepharoplast. At the same time I can quite well imagine that in other species the process may take a different course. It is seen that even in T. grayi the splitting does not extend to the whole length of the parent flagellum. In another species the amount of splitting might be much less, or might not extend beyond the division of the blepharoplast. I do not wish, at present at least, to extend my statements as to the splitting of the flagellum beyond the case which I have studied. That the flagellum does actually split in T. grayi is shown, I think, by the fact that in the early stages the daughter flagellum is distinctly finer and more delicate than the parent flagellum (figs. 205–208). I see no reason why this should be so if the daughter flagellum grew out independently from the blepharoplast.

The process of division described in the foregoing paragraphs is that most usually found, and represents, I believe, the normal course of events. The commonest departure from this course is seen in the division of the flagellum, which may be hastened and be completed before the division of the nucleus (fig. 213), or even before that of the kinetonucleus (fig. 215). This variation shows clearly that the flagellum is independent of the kinetonucleus, a fact which, in my opinion, is a further indication that the kinetonucleus should not be confounded with a true blepharoplast or centrosome.

(2) The slender type (figs. 144, 145, 172, 224–226, etc.), is very uniform in its characters and exhibits but slight variation in contrast with the many forms which I have grouped together as the "ordinary" type. The body is elongated and slender; the cytoplasm is free from coarse granules as a rule, but occasionally a few are seen in the posterior part of the body; *N* is compact, sometimes very compressed; *n* is large, rod-shaped or round, generally filling up the whole width of the body, and invariably in front of *N*; the flagellum is distinct and stains deeply; it stands off but little from the body, forming a very shallow undulating membrane, which is scarcely or not at all pleated, and the free flagellum is very long, and often appears distinctly thickened at its free termination.

The chief variation exhibited by this type is seen in the degree of slenderness of the body; usually slender, with a compressed nucleus (figs. 225, 226), it is sometimes stouter, with a round or oval nucleus, thus showing an approach to the ordinary young forms (fig. 224, etc.). In one instance (Nov. 10th, 1st fly, p. 241) I have found such moderately slender forms connected by transitions with round forms (figs. 132–137). Only in a single instance have I found what appears to be division; but I am by no means certain that this is not really a case of two trypanosomes accidentally superposed (fig. 147).

The slender type is distinguished in life by its extreme motility. The trypanosomes are seen darting across the field of the microscope in the manner aptly termed "en flèche" by French writers. The body is held stiff and moves with the flagellum, vibrating rapidly in advance. These are the forms termed by Novy (**32**) and by Gray and myself (**29**) "male" forms; I shall consider the point more in detail presently.

As a special development of the slender type should be reckoned, perhaps, the immensely elongated forms seen in figs. 242–244. Unfortunately I have not noted the region in which they occur. A similar form has been figured by Gray (P. R. S., B 78, Pl. XIII, fig. 33). My first impression of these remarkable forms was that they were simply spermatozoa of the fly, but they are connected by transitions with more ordinary forms.

(3) The Herpetomonas-type is to be regarded as a special modification of the slender type, though probably originating directly, in some cases, from ordinary young forms. The H-forms (as I may term them for brevity's sake) are minute and very slender, with the chief bulk of the body behind the kinetonucleus, in front of which the body tapers rapidly to a filamentous prolongation, sometimes of considerable length (figs. 163, 164, 169, 170, 184, 185, 187, 188, etc.). Posteriorly the body is usually bluntly pointed. The nucleus has the form of a clump of granules about the middle of the body. The kinetonucleus is invariably in front of the nucleus. The cytoplasm is clear, but sometimes contains a few coarse

granulations, which frequently occur in front of the kinetonucleus. The undulating membrane is sometimes distinct (fig. 163), but as a rule is rudimentary or absent. The flagellum is very long and very slender and delicate; it stains feebly or not at all, and is often difficult to trace. A typical example of this type is unmistakable, and contrasts sharply in many points with the slender type described above.

The H-forms have been found by me becoming encysted in great numbers in the proctodæum of one of my flies (Nov. 14th, p. 246). I have two smears of this specimen; one of them is a thin smear, the other much thicker. Both the smears show all stages of the encystment in abundance, but in the thin smear the cyst-wall, when developed, is nearly always more or less damaged, being evidently of soft consistence. In the thick smear the cyst-wall is usually intact and perfect, but the finer details of the contained body are not so well shown. Figs. 186–197, 202, 203 are drawn from the thin smear, as were also the figures published by me in my preliminary communication to the Royal Society (P. R. S., B 79, p. 37). Figs. 198–201 are from the thicker smear, which I had not examined at the time of making my preliminary communication.

My preparations of the proctodæum show, in the first place, a certain number of young forms of the ordinary type (figs. 183, 186). Similar young forms are also found as the preponderating type of trypanosome in the hinder part of the intestine of the same fly (p. 245), a fact of interest as indicating that the H-type arises from the young forms of the ordinary type. In the second place the smears show a large number of the H-forms (figs. 187, 188), which are also found sparingly in the hinder part of the intestine. In life they were observed swimming freely, and also occurring in masses attached to the wall of the proctodæum. In the third place there are the various stages of encystment, from the earliest modification of the H-form to the ripe cyst in its final form (figs. 189–202). The cysts at all stages were free in the lumen of the proctodæum, and not attached in any way to its walls.

The first sign of encystment is a shortening of the flagellum, which at the same time appears to become thicker and more distinct, an effect perhaps largely due, however, to its being difficult to distinguish the exact limits of the flagellum from the slender anterior prolongation of the body (compare figs. 189, 192). While the flagellum is becoming retracted the cyst-wall begins to appear round the hinder end of the body (figs. 189–193) in the form of a granular secretion, which stains a dull red with Giemsa's stain. The rate at which the cyst is formed shows great variations relatively to the retractation of the flagellum. In some, with the flagellum still long, an abundance of the cyst secretion is seen (fig. 190). In others, with the flagellum almost completely retracted, the cyst is only just beginning to be formed (fig. 193). The substance of the cyst-wall appears to be made up of distinct masses or grains of the red-staining substance, between which is a more fluid matrix, and appearances are often obtained very similar to those figured by Prowazek for Herpetomonas muscæ-domesticæ (**35***a*). The term "Schleim-cysten," applied by Prowazek to the cysts of Herpetomonas, appears suitable for the present case also, as the frequency with which burst or damaged cysts are met with (figs. 192, 195, etc.) indicates that the substance of the cyst-envelope is of a soft nature.

While the cyst-wall is being secreted the retractation of the flagellum is proceeding, until all that can be seen of it is a round or oval red-staining mass, connected by a red streak with the blepharoplast (figs. 194, 195). The appearances seen at this stage (which is of very common occurrence) are remarkable, and suggest strongly the "flagellar vacuole" described by Leishman as giving rise to the flagellum in the flagellated culture-form of Leishmania. Here, however, the flagellar vacuole, if it may be so termed, is, in its relation to the flagellum, the inverse of that of Leishmania—that is to say, it does not precede its formation but results from its retractation. The flagellar vacuole disappears and only the streak is left (fig. 196); finally the streak fades away too, and the retractation of the flagellum is complete. Meanwhile the cyst-wall, which made its appearance as a cap round the posterior end of the body, is secreted more and more towards the anterior end, and when the flagellum has completely disappeared, the cyst is formed round the anterior termination of the body, thus producing the characteristic pear-shaped cysts which are very abundant in the preparation (fig. 198). This appears from Prowazek's description to be the final stage of the cyst in the case of Herpetomonas; not so in T. grayi. The cysts, at first pear-shaped, with unlike ends, become more oval, with ends alike or scarcely distinguishable (figs. 199, 200), and finally they become more or less circular in outline, with the wall of even thickness all round (fig. 202). This stage is apparently the ripe cyst, and the last stage that can be observed in the body of the fly.

While the process of encystation is going on, noteworthy changes are taking place in the nuclei. The trophonucleus becomes resolved into chromidia. Usually one large, irregular mass of chromatin can be seen, together with a variable number of irregularly scattered chromatin grains (fig. 197). At the same time the kinetonucleus diminishes in size, apparently also as the result of fragmentation. Amongst the chromatic grains scattered in the body, some can be distinguished by their reddish colour, like that of the nucleus, others by their purple colour, like that of the kinetonucleus (fig. 195); the latter are fewer in number. It is important to note that the cyst substance also stains a reddish tint, similar to that shown by the granules of the nucleus, and it is possible that the disruption of the nucleus is in relation to the secretion of the cyst.

In the ripe cysts the kinetonucleus seems to disappear completely. In some of them it can still be made out plainly (fig. 202). In others it cannot be identified with certainty, but amongst the chromatic grains some can be seen which exhibit a more purple tinge than the others, and which represent probably the kinetonucleus broken up. The significance of these changes could only be made out by studying the germination of the cyst, and that I have not been able to do.

In a few rare cases I have observed division within the cyst; one such case is figured (fig. 203).

Having, in the foregoing, described the various forms under which I have met with Trypanosoma grayi in Glossina palpalis, I propose now to discuss the significance of the different forms and their relations to one another. This is not an easy matter in the incomplete state of our knowledge of the life-cycle of this trypanosome. Moreover, the forms which T. grayi assumes are so different that they might well be taken for different species were it not that they are all linked by transitions. Taking first the forms which I have grouped together as the "ordinary" type, we may, I think, regard them as the multiplicative form of the trypanosome in the fly, that is to say, the form which, when taken from the vertebrate host, has the function of feeding, growing, and multiplying by division to produce the vast swarms of trypanosomes which are found in infected flies. I base this opinion on the following grounds: First, the large size which this form reaches, a character not likely to be exhibited by forms destined to pass back from the fly to a vertebrate host; such forms are more likely to be of small size; secondly, the frequent occurrence of division and production of young and intermediate forms; thirdly, the great variability of this form, especially in the position of the kinetonucleus. Far from being constantly in front of the nucleus, the kinetonucleus may be behind it or even terminal. From our knowledge of other trypanosomes, I think we may assume that the forms with *n* terminal probably represent most nearly the form under which T. grayi occurs in the vertebrate host. It is true that it is not even known that this trypanosome has a vertebrate host. I have elsewhere (P. R. S., B 79, p. 38) brought forward arguments to favour the belief that T. grayi has a vertebrate host, and is not simply a parasite of tsetse-flies, and in my opinion the occurrence of a form with *n* terminal, like an ordinary blood-trypanosome, is still more in favour of this belief. I would add, further, that from the conditions under which T. grayi was found to occur in one of my flies (Oct. 10th, p. 232), I am of opinion that the host of T. grayi is a bird, probably some of the numerous species of diving birds which swarm on the shore of the Victoria Nyanza.

We have next to consider the slender forms, formerly regarded by me as male. I do not think this interpretation can be maintained, in view of their distribution and mode of occurrence in the fly. Thus, in one of my flies (Nov. 10th, [1], p. 241), only slender forms, round forms, and H-forms occur, and are confined to the proctodæum (figs. 132–137). Some of them are fairly stout and approach young or ordinary forms in character, but with *n* constantly anterior to *N*. In Nov. 10th (2) also we find most typical slender forms in the proctodæum, but none in the intestine, where ordinary forms of all kinds occur (p. 242, figs. 138–150). In Nov. 13th we find no slender forms in the stomach, but in the intestine and proctodæum we find both slender forms and typical H-forms mixed with ordinary forms (figs. 156–170, p. 243). In Nov. 14th we find slender forms very sparingly in the stomach and intestine, while the proctodæum contains swarms of H-forms and cysts (figs. 171–216, p. 245). Finally, in Oct. 10th we find slender forms present fairly commonly in the stomach and intestine, together with ordinary forms, but in the proctodæum we find immense numbers of the slender and H-forms, without any ordinary forms (figs. 218–233, p. 233).

The data given above show that the primary habitat of the slender type is the proctodæum, of which it may constitute the sole trypanosome-fauna; and further that all other forms may be absent altogether from the fly. This, I think, sufficiently disposes of the notion that the slender form represents the male type. From the proctodæum both the slender form and the H-form may extend forwards; it is noteworthy, however, that the slender form gets further forward, being found in the stomach, while the H-form does not, so far as my observations extend, get further forward than the hinder part of the intestine. It would be interesting to know if the slender forms penetrate as far as the proventriculus. Unfortunately none of the flies examined by me had trypanosomes in the proventriculus.

The slender form and the H-form are in all probability to be regarded as propagative forms, that is to say, as forms destined to spread the species to other hosts. In favour of this view may be urged their small size and great activity, the absence of multiplication amongst them, their mode of occurrence in the fly, and their constant structure, far removed from that of the ordinary blood-trypanosome and more approaching the Herpetomonas or Crithidia type of structure, that is to say, the type of structure usually associated, in these organisms, with parasitism in the gut of insects. The H-form becomes encysted in the proctodæum, and the cysts are doubtless destined to pass out with the fæces. What is the destiny of the slender type? The manner in which it extends forwards in the gut suggests strongly that it may be destined to infect fresh hosts by the inoculative method.

To sum up my conclusions as to the various forms of Trypanosoma grayi: They may be subdivided at the outset (using Doflein's appropriate terms) into (1) multiplicative forms, varying greatly in size and structure; and (2) propagative forms, very constant in both respects, but of two types. One of the propagative types terminates its lodgment in the fly by becoming encysted in the hind gut; the other is perhaps destined to pass forwards through the proboscis.

If the two types hitherto regarded as male and female be not in reality such, the question arises whether any of the numerous forms of T. grayi are to be regarded as sexual. It is usual, when slender and stout forms of trypanosomes occur together, to interpret them as male and female; but the only final and conclusive proof of such forms being sexual in nature is to find them conjugating. I have spent much time trying to find stages of conjugation in my slides of T. grayi, but among the many thousands of trypanosomes that have passed under my vision I have found none that could be interpreted with certainty as conjugating forms, and very few which could be suspected of this. Since conjugating forms might conceivably be confused with dividing forms, I studied in detail, and have described above, the process of division and its variations, which are chiefly variations in the rapidity, relative to one another, with which the flagellum and the two nuclei (*n* and *N*) divide. I have found a few specimens which might perhaps be conjugating forms, and have figured three of them. In one (fig. 214) it is seen that the two flagella are widely separate, that there are two large kinetonuclei in close contact, and that there is a single compact nucleus, very dense in texture. If this be a variation of division, it is a remarkable one, not only in the very precocious division of the flagellum, and in the fact that the body has begun to divide before the nucleus has divided, but, above all, in the very large size of the two kinetonuclei; I have never seen any division-stage in which the daughter-kinetonuclei were of such large size. On the other hand, this specimen would very well bear interpretation as a fusion, which had commenced from the hinder end of two individuals, in which the bodies are nearly fused, the nuclei completely so, the kinetonuclei are beginning to unite, and the flagella are still quite separate. If this interpretation be the right one, fig. 216 might possibly represent a further stage of the same process. Another possible instance of conjugation is seen in fig. 143, in which the kinetonuclei are united, while the nuclei are distinct. If these three figures really represent instances of conjugation,[1] they do not throw much light on the characters of the males and females, except to show that one partner has a long free flagellum, the other a short one. I am inclined to identify the two varieties of the ordinary form, termed above the serpentine and the vermiform types, as male and female respectively, and fig. 143 supports this view; but it must be confessed the evidence is meagre. Some of the large ordinary forms (figs. 138, 139) might then be really zygotes.

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[1] The forms figured by Stuhlmann (**41**) and interpreted by him as conjugation-stages are very different in appearance, especially in having the two flagella on the outer sides, furthest from each other, instead of close together. Stuhlmann considers the position of the flagella diagnostic of conjugation as compared with division; this makes it still more doubtful if the forms figured by me are really conjugation-stages.

A few words, in conclusion, upon the differences between T. grayi and T. gambiense and the nature of the former. As a rule the two forms can be distinguished at a glance, whether in the living condition or in preparations. T. gambiense is sluggish in its movements, and when observed in vitro seldom moves out of the field. T. grayi, on the contrary, moves with great rapidity, and is very difficult to keep in the field. The slender forms, in particular, dart across like arrows. But in two flies containing T. gambiense seventy-two hours after infection, I observed that the trypanosomes were very active in their movements (Oct. 22nd, p. 237), and in the preparations very large trypanosomes were found.

The most constant distinctive feature of T. grayi is its large rod-shaped kinetonucleus. In T. gambiense *n* may be round or rod-shaped, but is always much smaller than in T. grayi. Moreover, in T. grayi, though the position of *n* is variable, it is most usually at the side of, or in front of, *N*, and when division is about to take place, *n* is always in front of *N*. In T. gambiense, so far as I have observed, *n* is always behind *N* by at least half the distance between *N* and the posterior end of the body. It is sometimes difficult to draw clear distinctions in words, but I think the figures show that it is impossible to confound T. grayi and T. gambiense at any stage. It is, nevertheless, interesting to note that in the two instances in which T. gambiense did not show any signs of degeneration on the third day in the fly, it made some approach to T. grayi in its appearance and activities. T. grayi, as seen in the fly, is probably a good deal different from the same species in the blood of the vertebrate host.

I think there can be no doubt that the forms described by

Koch (**20**) as developmental stages of T. gambiense were really T. grayi or a similar form.

With regard to the origin of T. grayi, our first notion was, as I have stated, that it was identical with T. gambiense; when this notion was dispelled I was at first inclined to regard it, with Novy, as a parasite of the fly itself, and my discovery of the encystation seemed to confirm this idea, which, however, more mature reflections, especially considerations of the habits of the fly, made me give up. In my preliminary report (P.R.S., B 79, pp. 38, 39) I have stated my reasons for believing that T. grayi has a vertebrate host, and I have nothing to add to them. For various reasons we suspected T. grayi to be an avian trypanosome, though we were not able to prove this, but Novy has shown that it is often impossible to find trypanosomes in bird's blood microscopically when their presence can be demonstrated culturally. The fact that a fly (Oct. 10th, 1905, p. 232), bred in captivity, becomes infected with T. grayi after feeding on fowls used to feed freshly caught flies, seems to me proof positive that T. grayi is an avian parasite[1]; but if so, I may point out as a corollary, it also suggests that T. grayi can be transmitted by the inoculative method.

IV. REMARKS ON THE LIFE-CYCLE AND MODE OF TRANSMISSION OF TRYPANOSOMES.

The scientific study of the transmission of trypanosomes, and their relation to disease, dates from the publication of Bruce's masterly reports on his investigations upon Nagana in Zululand in 1895 (**3**, **4**). It is not necessary for me to dilate at length upon the results, well known to everyone, of these researches, admirable alike for their conception, execution and presentation, and marking, as Koch has well said, the beginning of one of the most important epochs in the study of the Protozoa.

The publication of Bruce's reports seems to have aroused almost immediately suspicions as to the true nature of sleeping sickness, since Blanchard (1) quotes a sentence from a memoir published in 1898, by J. Brault, in which the opinion is expressed that sleeping sickness is a protozoal disease caused by trypanosomes and transmitted by tsetse-flies. The news, however, of the discovery of trypanosomes in the cerebro-spinal fluid of sleeping-sickness patients, by Castellani in 1903, produced quite a chorus of prophetic utterances, predicting or arguing that sleeping sickness would prove to be transmitted by tsetse-flies. According to Blanchard, this view was expressed by himself on June 18th, by Brumpt on June 27th, and by Sambon on July 1st, 1903, in each case independently of the others. Let who will, however, claim the gift of prophecy or the talent for drawing reasonable inferences from the analogy of established truths, it was nevertheless Bruce, who, in collaboration with his colleagues of the Sleeping Sickness Commission, Nabarro and Greig, first supplied the experimental demonstration of the transmission of sleeping sickness by tsetse-flies. All later observations have but confirmed the statements of these pioneer investigators, without so far adding anything of importance to their results. That Glossina palpalis can and does convey the infection of sleeping sickness, may, I think, be taken as an established fact; and up to the present no other method of infection has been proved to exist. This brings the etiology of sleeping sickness into line with that of other trypanosome infections, all of which, so far as present knowledge extends, are transmitted only by the intermediary of blood-sucking invertebrates, with a single exception—the well-known case, namely, of dourine in horses, a disease known to be transmitted from sick to healthy animals by coitus. This exception to the general rule is of considerable interest, as showing that, in this case at least, the trypanosomes have the power of passing through mucous membranes.

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[1] Koch seems inclined to consider the crocodile as the vertebrate host of T. grayi or of other tsetse-fly trypanosomes.

There remains, however, the question of the exact manner in which the infection is transmitted by means of the blood-sucking invertebrate. In considering this question, the facts known with regard to malaria rise at once to the mind. The researches of Ross, Grassi, and others have demonstrated that in malaria the mosquito is a true host in which the parasite goes through a complicated developmental cycle, at the end of which, and not before, the mosquito is able to infect a fresh vertebrate host with the parasite. A mode of infection of this kind may be termed conveniently a cyclical type of infection, and, since it is effected by the mosquito inoculating healthy subjects with the parasites, we may further characterise it as the inoculative cyclical type. It is an obvious suggestion, from the analogy of malaria, that trypanosomes may also undergo a cycle of development in their invertebrate hosts, a suggestion that received, apparently, concrete proof in the well-known investigations of Schaudinn (**40**) upon Trypanosoma noctuæ. But it is becoming increasingly evident, I think I may say, that Schaudinn's statements with regard to Trypanosoma noctuæ must be regarded with scepticism until they have received confirmation, in view of the many possible sources of error from mixed infections which his material presented. Nevertheless, Schaudinn's results have been regarded by many as conclusive proofs that trypanosomes pass through a developmental cycle in their invertebrate hosts. I am conscious myself of having gone to Uganda to investigate this question with a distinct bias in my mind, believing that the infection of sleeping sickness would prove to be of a similar type to that known in the case of the malarial parasite, and stated by Schaudinn to occur in T. noctuæ—namely, the cyclical inoculative type.

On the other hand, many experimental results, so far obtained, indicate that the mode of transmission of sleeping sickness, and perhaps of other trypanosome infections also, is not of the type of malaria, but is in many cases, at least, a direct one—that is to say, that the trypanosome does not go through a developmental cycle in the invertebrate host, but is inoculated mechanically by the proboscis of the blood-sucking intermediary. Thus Brumpt, in 1903 ([**7**] p. 1497), expressed the view that the rôle of the tsetse-fly could not be compared to that of Anopheles in malaria, since the tsetse transmits only for forty-eight hours, and no longer. At the present time authoritative opinion on the subject may be said to be divided into two camps, some denying the existence of cyclical infection, others believing it to occur. An extreme example of the former school of thought is Novy, who is of the opinion that the insect plays but "a passive or mechanical part" in the transmission of trypanosomes ([**33**] p. 13 of reprint), and who has cast grave but reasonable doubts upon the correctness of Schaudinn's famous investigations upon Trypanosoma noctuæ.

Confining our attention, for the moment, to the transmission of sleeping sickness, that is to say, of T. gambiense by Glossina palpalis, we have, in the first place, the transmission experiments carried out by Bruce, Nabarro, and Greig (**6**), which can be most conveniently summarised in a tabular form :

Number and reference of experiment.	Number of flies fed on patient.	Flies fed on healthy monkey.	Interval between the two feeds.	Infection produced after—
114 (loc. cit., p. 57) . .	880	530	8 hours	65 days
115 (loc. cit., p. 58) . .	881	509	8 ,,	65 ,,
99 (loc. cit., p. 59) . .	582	508	24 ,,	70 ,,
97 (loc. cit., p. 60) . .	294	255	24 ,,	48 ,,
116 (loc. cit., p. 61) . .	354	267	48 ,,	65 ,,

These experiments prove the transmission of the trypanosome of sleeping sickness by G. palpalis, and indicate that the transmission is a direct one, since with a longer interval than forty-eight hours no infection was obtained. I say "indicate," because the fact that freshly-caught flies were used for the experiments invalidates conclusions as to the exact method of the infection, since it was also proved by the same investigators that freshly-caught flies may produce infection with trypanosomes, without having been fed previously upon infected animals. Thus, in one case a monkey became infected when 216 freshly-caught flies had been fed on it in batches over a period of fifteen days (loc. cit., p. 62); in a second case 894 flies produced an infection after twenty-eight days (loc. cit., p. 62); and in a third case, 759 flies infected after twenty-three days (loc. cit., p. 63). To these three cases may be added two experiments recorded by Greig and Gray (**18**); in the first 980 flies produced an infection after forty-six days (loc. cit., p. 106); in the second, 2299 flies were fed on a monkey over a long period without infecting it. Finally, reference may be made to the result obtained by us (**29**, p. 245), in which a batch of 134 flies fed on a monkey (as a control experiment) produced an infection in it. If we add all these results together we find that a total of 5282 tsetse-flies, freshly caught in the neighbourhood of Entebbe, infected five animals; but if we take separately the experiments of Bruce, Nabarro, and Greig (**6**), made at a time when remedial measures had not been undertaken at Entebbe, a much higher average is the result, since we find that 1869 freshly-caught flies produced three infections, an average of one infection for 623 flies, which is a number very little higher than that of the flies which were operative in the eight-hour transmission experiments. There was, therefore, the possibility that the infections produced in the transmission experiments tabulated above were not really produced by transmission from the infected to the healthy subject, but by flies which had been in an infective condition when first caught, before being used for the experiments. It is noteworthy also how much more quickly, in all the successful experiments, the infection was produced with the freshly-caught flies, so that it would appear to be almost more dangerous to be bitten by free flies in a sleeping sickness locality than by those artificially infected in the laboratory.

A further point to note in the experiments tabulated above is the relatively large number of flies required to produce infection at the short intervals (eight hours). I believe this is to be accounted for as follows: when a batch of flies has been fed and is put on a second animal eight hours later, the majority of flies being gorged with blood do not feed a second time; the flies that feed at the second opportunity are probably those which did not feed on the first occasion. It is comparatively rare for a fly not to gorge itself the first time it is put on to an animal.[1]

For these reasons we sought to obtain evidence more decisive as to the exact nature of the transmission. As stated in our preliminary report (**29**, p. 244), we took one fly at a time, fed it partly on the infected animal, and then transferred it to the healthy animal. In this way we infected[2] nine out of ten animals each with a single fly, which may, I think, be taken as conclusive proof of the transmission being direct, and not due to any pre-existing infectiveness of the flies used, since the proportion of infective flies amongst those caught while near Entebbe was at that time probably less than one in a thousand. It is true that these experiments were done with the "Jinja" strain of trypanosome (a further proof that it was not due to any previous infection of the flies used in the experiment); but I think it may be claimed that they prove what they were intended to prove—namely, that the tsetse-fly can, and does, effect direct mechanical transference of trypanosomes from an infected to a clean animal by means of its proboscis.

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[1] I have not made detailed reference to the experiments of Dutton, Todd, and Hannington (**15**), as I wish to confine myself more especially to the region in which I worked. The investigators named seem to have been singularly unsuccessful in their experiments, only four (three with T. gambiense, one with T. dimorphon) giving a positive result out of a total of thirty-nine. It is noteworthy that in three out of their four successful experiments the animal that became infected had been bitten by Glossina fusca as well as G. palpalis. Cazalbou (**14**) seems to have been more fortunate in his experiments with freshly caught G. palpalis. Dutton, Todd, and Hannington consider it "certain that . . . mechanical transmission cannot be the only way in which Trypanosoma gambiense is transmitted from man to man" (loc. cit., p. 213).

[2] Thus confirming a suggestion of Bruce (**4**, p. 3) who wrote, with reference to nagana, "I have no doubt one fly would give the disease if taken while feeding on an affected animal and placed straightway on a healthy one."

To prove direct transmission, however, does not disprove the existence of an indirect or cyclical method. It is logically impossible to prove a universal negative since any such proposition is invalidated by a single instance to the contrary. We can only say that our attempts to obtain evidence of a cyclical infection gave negative results. Such attempts were of two kinds: first, experiments to show periodicity in the effectiveness of the flies, such as is known to occur in the case of malaria; secondly, observations on the fate of T. gambiense when taken up by the fly. By experiment we obtained infection up to, but never after, forty-eight hours from the time that the fly fled on the infected animal. By observation, as already stated at length in this memoir, it was found that T. gambiense at first multiplied and became differentiated in the gut of Glossina palpalis or other Diptera, and also increased continually in size, but sooner or later died out and could never be found in G. palpalis on or after the fifth, sometimes not on the fourth day after infection. In all forty-two tsetse-flies have been examined by me at ninety-six hours or later after feeding on infected animals, with invariably negative results, although all the flies from the same batches were found to contain T. gambiense more or less abundantly when dissected at earlier periods.

Very different results from those obtained with sleeping sickness are furnished by the recorded experiments and observations with regard to nagana, the tsetse-fly disease of animals caused by T. brucii. If we take first of all Bruce's experiments on the transmission (**3**) we are struck at once by the much smaller number of flies used to produce a result. Where hundreds were required for an infection with sleeping sickness, tens or even units were sufficient for an infection with nagana. Thus in one experiment (228, loc. cit., p. 5) eight flies, fed four times on a healthy dog immediately after feeding on an infected one, produced an infection; the same result was attained in another case (Experiment 222, loc. cit., p. 5) with eight flies after only three feeds; with twenty-four hours' interval (Experiment 291, p. 6), nine flies produced an infection after thirty-eight days, and again, with the same interval (Experiment 232, p. 6), twelve flies infected a dog in the same number of days; with forty-eight hours' interval (Experiment 317, loc. cit., p. 7) seventy flies fed in batches over thirty-one days were required, but longer intervals were not successful. With freshly-caught flies, one hundred and twenty-nine flies, fed in batches, infected a horse after twenty-four days (Experiment 225, loc. cit., p. 15), and ninety-eight flies infected a dog after nineteen days (Experiment 236, p. 16). If these experiments favour the view that the infection is a direct one, they at least indicate that infection of this type is much more easily obtained in this way with T. brucii than with T. gambiense. There is, however, in one of Bruce's experiments a circumstance which, taken in connection with an experimental result of ours, seems to me to show clearly that the infection of T. brucii was not always of the direct type. Bruce's Experiment 225 (loc. cit., p. 15), to which reference has been made already, was carried out to answer the question, "Is the tsetse-fly capable of giving rise to the disease if taken out of the fly country into a healthy locality?" In the account given we read that "the method of carrying out this experiment was to go down to the fly country in the early morning, catch the flies, return to the top of the Ubombo, and straightway place them on the animal under experiment. The greatest care was taken that the flies were caught on a perfectly healthy animal, as to have allowed them to puncture one already affected by the disease would naturally vitiate the experiment." Now in our experiments on direct transmission, already recorded (p. 244), we found that if the fly, after feeding on an infected animal, were fed on two healthy animals in succession, only the first healthy animal became infected, not the second—that is to say, that by puncturing the skin of a healthy animal the proboscis is "cleaned" for a second one. Hence, if the infection of T. brucii were only by the direct method, the flies caught off a healthy animal, in Bruce's experiment, should have been non-infective. The experiment seems to me, therefore, to indicate that in the case of T. brucii there is infection of a type other than the direct—that is to say, that cyclical infection occurs, doubtless in addition to direct infection.[1]

Turning from experiment to observation, we have the results obtained by Koch (**19**) and Stuhlmann (**41**), who have studied the development of T. brucii in Glossina fusca, and have found a state of things which are in sharp contrast with my results for T. gambiense and G. palpalis.[2]

Koch (**19**) found a multiplication of the trypanosomes, with differentiation into stout and slender forms; next, multinuclear forms, believed to be zygotes, from which arose round forms, which in their turn gave rise to small forms with *n* in front of *N*. Long narrow forms, with *n* far in front of *N*, were also found. In the proboscis fluid trypanosomes were found resembling blood-trypanosomes in form and size, in addition to other forms. Attempts to infect rats with the trypanosomes in the digestive tract of tsetses were always without result. Koch gives reasons for believing that the infection produced in the fly differs according to the state of the trypanosomes in the blood of the sick animal; in flies fed on animals freshly infected, with many trypanosomes in their blood, development was not obtained, but in flies fed on sickly animals with few trypanosomes in their blood, a vigorous growth and development of the trypanosomes resulted.

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[1] The great infectiveness of tsetse-flies in the case of nagana is notorious. According to the unanimous testimony of travellers, confirmed by Bruce's observations, it is only necessary for susceptible animals, such as horses or cattle, to pass through the "fly-belts," in order to acquire the disease, practically as a certainty. This is not the case in sleeping sickness. In Uganda I was struck not so much by the number of sick persons in affected areas as by the number of healthy persons living under precisely the same conditions. The infection of sleeping sickness by the bites of tsetses may be compared to a lottery, in which the prizes are few and the blanks are many.

[2] Bruce (**4**) found, in tsetses infected with nagana, trypanosomes in the stomach up to the end of the fifth day, so long as any blood remained undigested.

Stuhlmann (**41**) has published a preliminary account of the results obtained by himself and Kudicke. These investigators worked both with freshly-caught flies and with flies bred in captivity. The latter were found to become most easily infected if fed on the infected animal for their first meal after being hatched from the pupa; in 80–90 per cent. of the flies so treated, after a short time (2–4 days) the intestine (Hinterdarm) became full of trypanosomes, indifferent forms showing many division-stages. Stuhlmann's figures of sections show the trypanosomes present in enormous numbers, similar, in my experience, to an infection with T. grayi. From the intestine the infection was found to spread forwards into the stomach (Mitteldarm), but rarely extended further forward than this region, unless the flies were re-fed on healthy animals; when that was done the infection could be traced forward as far as the proventriculus. Infection of the proboscis was not produced in artificially infected flies, but was found in a small percentage of those naturally infected. It was found, moreover, that in artifically infected flies the infection produced in the intestine often gradually died out, and only about 10 per cent. of the experimental flies obtained an infection of the stomach and thoracic intestine; thus the percentage of flies that became thoroughly infected in the laboratory came to about the same as the percentage of flies found infected in nature. The authors figure forms which resemble to a certain extent some forms of T. grayi, but which are characterised by a small round kinetonucleus. They distinguish indifferent forms, long forms, and small forms. The long forms are found in the proventriculus and œsophagus, rarely in the proboscis. The small forms are seen, from the figure given (loc. cit., fig. 158 *a*), to be very similar to the small forms of T. grayi with *n* in front of *N* (fig. 182) except for the characteristic difference of the kinetonucleus. It is remarkable, therefore, that the authors should find the small forms almost exclusively in the proboscis, seldom in the gut, even in its most anterior parts; for, as I have described above, the corresponding forms of T. grayi are, on the contrary, most abundant in the hindmost parts of the digestive tract. Amœboid forms, with flagella reduced or absent, are also described, and are stated to be present sometimes in great numbers, but only in hungry flies. They are regarded as resting phases, and perhaps correspond to the round forms of T. grayi, but I have never seen anything like the "giant amœboids, with 4–16 nuclei," figured by Stuhlmann (loc. cit., fig. 154).

The following is the scheme of development suggested by Stuhlmann: The infection begins by a multiplication of indifferent forms in the intestine. From this point they spread forwards as far as the proventriculus, where Stuhlmann has seen and figured (loc. cit., fig. 152 *a*—*g*) appearances which he believes to be conjugation. After conjugation, probably, are produced the small forms, which may be the infective forms which pass back into a new vertebrate host. The nature of the long, slender forms is doubtful; they appear simply to die out. In no cases were trypanosomes found in the end gut (proctodæum), nor in the salivary glands, nor in any organs of the fly except the proboscis and the digestive tract proper—that is, as far back as the end of the intestine.

The fact that Stuhlmann and Kudicke used only flies bred in the laboratory for their infection experiments may be taken as proof that the trypanosomes observed by them in these flies were truly stages of T. brucii. Stuhlmann is of opinion, and I agree with him, that trypanosomes are not transmitted by tsetse-flies to their posterity; in other words, that so-called germinative or hereditary infection does not occur.[1] It is evident, then, that T. brucii not only multiplies but goes through a developmental cycle in G. fusca, ending with Herpetomonas-like forms, probably destined to infect vertebrate hosts. These results are entirely different from those obtained by me with T. gambiense in G. palpalis, where I found, without exception, that the trypanosomes did not survive after the fourth day, did not extend forwards into the proventriculus, and did not produce forms with *n* in front of *N*. As compared with T. grayi, the most noteworthy point of the development of T. brucii is that the "propagative" Herpetomonas-like forms occur only in the proboscis or most anterior part of the gut. In T. grayi, on the contrary, they are most abundant in the hindermost part of the gut, where they may become encysted. This difference between the two forms indicates that in T. brucii there is only probably inoculative infection, and no encystment or contaminative infection.

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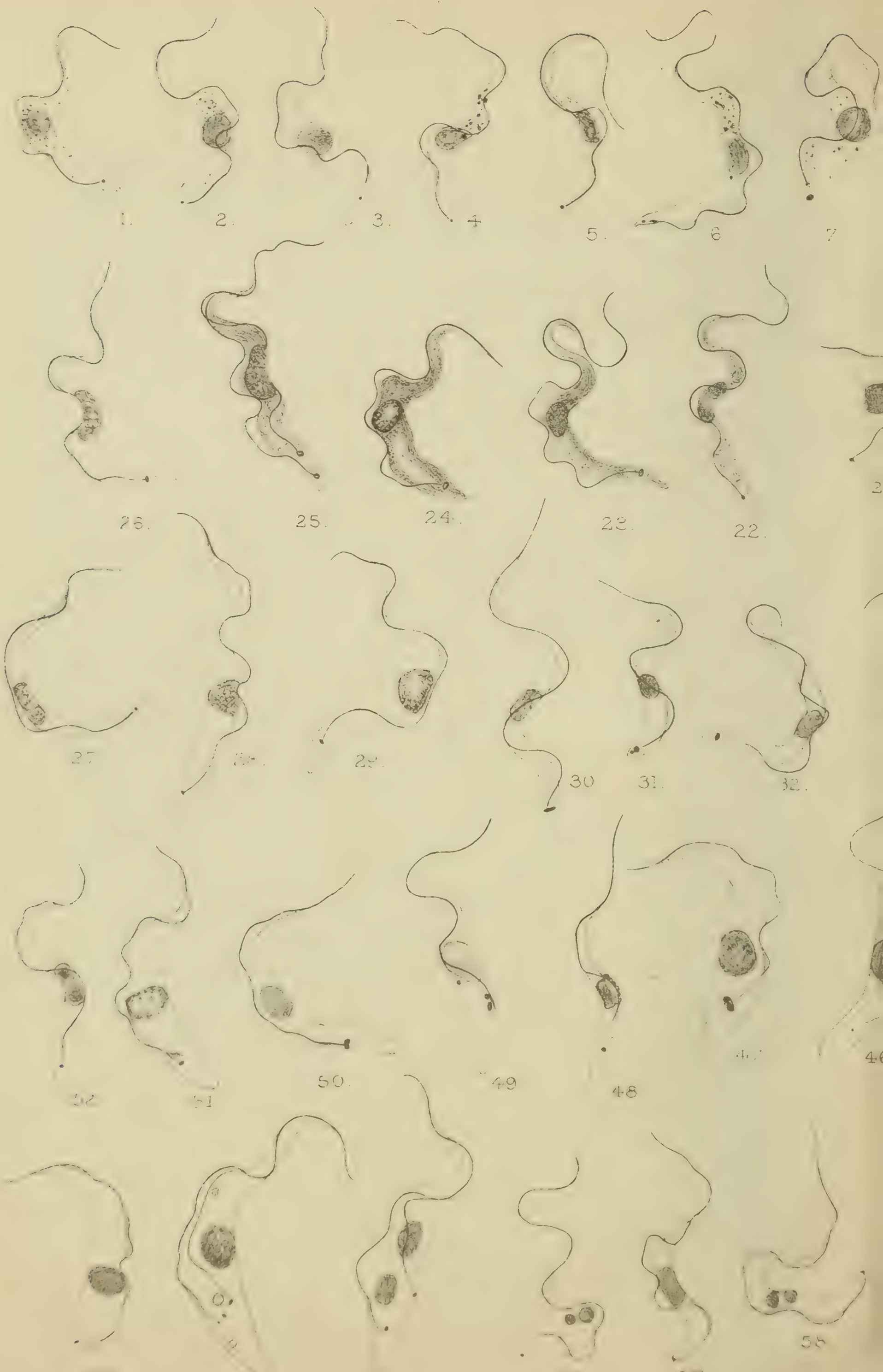
[1] I cannot follow Novy (**32**) when he refers to the supposed occurrence of trypanosomes in tsetses that have never fed on animals; he even commits himself to the extraordinary statement that "only a very small percentage of biting insects ever feed on blood" (loc. cit., p. 406). I can only explain such an utterance on the part of an investigator so skilled and experienced by

From the results, therefore, both of experiment and observation, it seems to me proved that T. brucii goes through a true developmental cycle in at least one species of the tsetse-flies that are agents in its transmission. Why, then,

his lack of acquaintance with the habits and life-history of tsetse-flies—a knowledge only to be acquired, of course, in Africa. From my own experience, I agree thoroughly with Stuhlmann (**41**, p. 44) that Glossina lives exclusively on blood and contains in its gut only organisms taken up with blood or derived from its mother. Our experience is that Glossina is a most greedy and rapacious bloodsucker; we have seen it feed readily on frogs, lizards, snakes, chameleons, crocodiles (compare Koch [**21**], p. iv), and birds, as well as mammals. On one occasion, a chameleon put into a fly-cage was at once attacked by them, and, being unable to defend itself, was so bitten that it died in twenty minutes from loss of blood. We also made many attempts to feed tsetses in other ways than with blood, but always without result (compare Stuhlmann, loc. cit., p. 44). On the other hand, there is not the slightest evidence that the tsetse can inherit trypanosomes from its parents (compare Stuhlmann, loc. cit., p. 70). Dutton, Todd, and Hannington (p. 202) hint at this possibility, solely from the far-fetched analogy of Piroplasma in ticks; but in this case it is known that the mother-tick passes on to each of her offspring a supply of undigested blood containing the stages of the parasite. It is not necessary to point out that Glossina differs totally in both habits and life-history from either ticks or mosquitoes.



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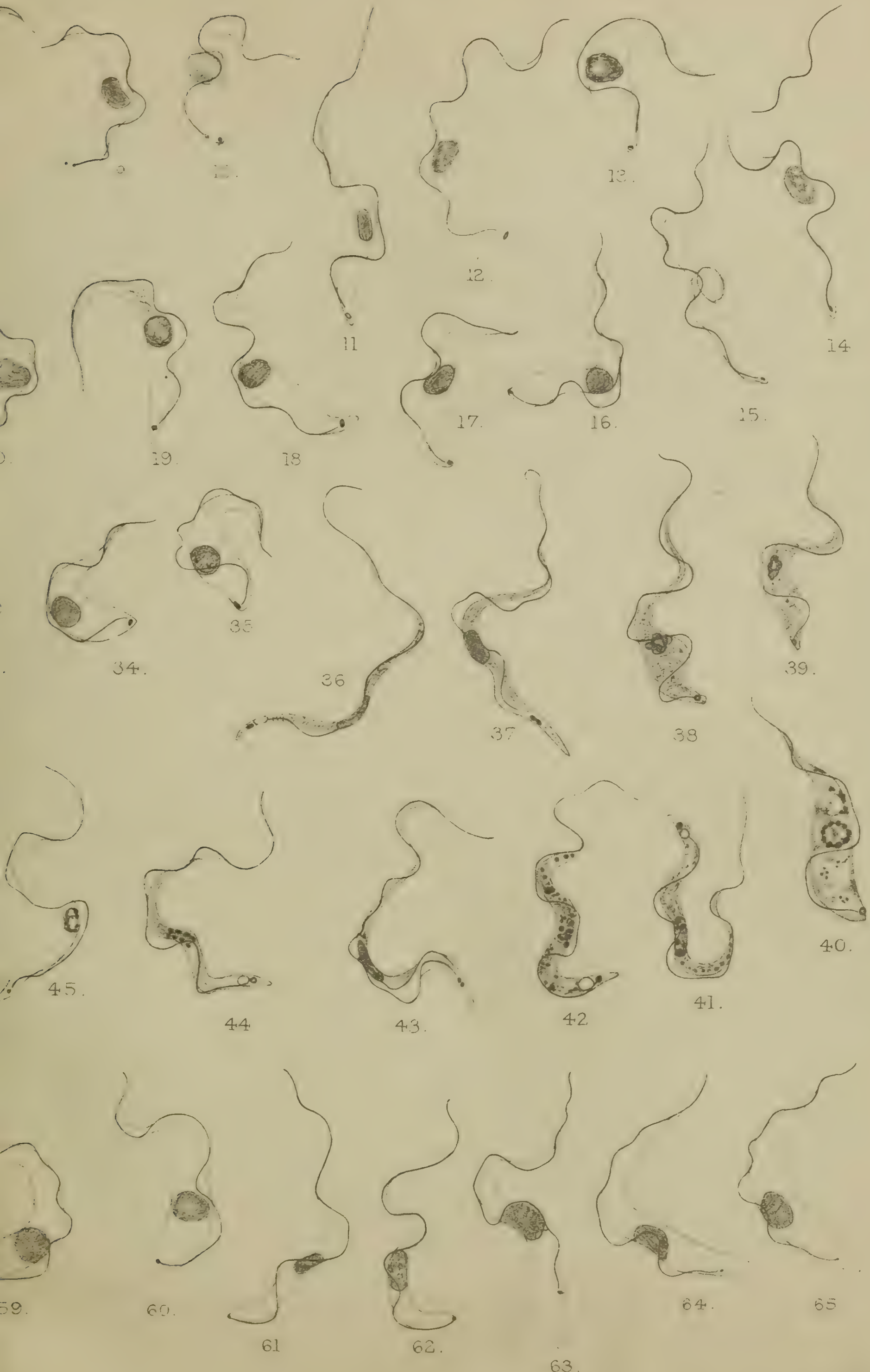

Huth Lith^r London

has T. gambiense given only negative results as regards a developmental cycle? If T. brucii is capable of developing, T. gambiense should be capable of doing so also. I can only explain my repeated failures to obtain development by the hypothesis that G. palpalis is not capable (in Uganda, at least) of acting as the true host of T. gambiense, but only of transmitting it by the direct method. Sleeping sickness, as is well known, has only been introduced into Uganda in comparatively recent times, having apparently come from regions further west. The appalling results produced by the epidemic brought about the appointment of our Commission, which carried on its investigations in Entebbe—that is to say, in a region where great mortality was taking place and the danger was most urgent. But in and around Entebbe G. palpalis is the sole species of tsetse-fly occurring, and has, therefore, obtained the sole credit of transmitting the disease which corresponds with it exactly in distribution. It is very possible that if our Commission had carried on its investigations in a region were more than one species of tsetse occurs, it would have been found that other species were equally effective. Brumpt, in 1904 (**8**), gave reasons for believing G. fusca to be also active in transmitting sleeping sickness.[1] But more decisive on the point is the observation of Koch (**21**), who fed forty-two Glossinæ fuscæ and eleven G. tachinoides, all of them young forms bred in captivity, on rats infected with T. gambiense, with the result that ten to twelve days later eight fuscæ and three tachinoides were found to be infected with trypanosomes that must be identified with T. gambiense, in view of the fact that virgin flies, as we may conveniently call them, were used for the experiment. No such result was ever obtained by me with G. palpalis; and I think it is a sound deduction from the observation of [illegible] with my own experience, that G. palpalis is not a suitable host[1] for the development of T. gambiense, though efficient in transmitting this trypanosome mechanically, while in other species of tsetses T. gambiense is capable of passing through a developmental cycle[2] To find support for this conclusion it is not necessary to rely solely on the analogy of the well-known facts of the transmission of malaria and mosquitoes. The interesting studies of Brumpt (**9**, **10**, **11**) on the transmission of fish-trypanosomes by leeches have shown that for a given species of trypanosome there is what may be termed a right leech, and that other species of leeches are wrong ones. Thus, for T. granulosum of the eel the right leech, according to Brumpt, is a Hemiclepsis, in which the development is completed, while three others, namely, Calobdella punctata, Hirudo troctina, and Piscicola geometra, were wrong leeches, in which only a part of the development was passed through. There is no reason to suppose that in the case of trypanosomes the same intimate relation between host and parasite does not obtain that is known to occur in the case of other parasites, protozoan or metazoan.

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[1] [illegible] ever, obtained "entirely negative" results with G. fusca, [illegible] hat there was "not sufficient material in these experiments to come to any definite conclusion."

Still less can I adhere to the peculiar view of Novy, that trypanosomes do not go through any life-cycle in their invertebrate hosts, but only a process of multiplication analogous to that seen in artificial cultures. Novy seems to regard the invertebrate host as nothing more, so far as trypanosomes are concerned, than a kind of flying culture-tube, an imitation of art by Nature; and, like some other writers, he does not seem to realise the essential distinction between multiplication and development. We can, perhaps, see in this attitude towards the problem the predisposition of an accomplished bacteriologist unaccustomed to think zoologically, if I may use such an expression. He expresses the belief that the trypanosomes found in biting insects are harmless parasites of the fly (p. 406) "derived from plant juices, stagnant waters, etc." (p. 404). In speculating about things unknown it is surely safer to reason from established data than from unfounded hypothesis. At the present time true trypanosomes are only known to occur in the blood of vertebrates, and in the stomachs of insects which suck the blood of vertebrates; hence, it is reasonable to assume that the insects in question obtain their trypanosomes from the vertebrates. When trypanosomes have been found in plant juices or stagnant waters it will be time enough to speculate on the possibility of blood-sucking insects obtaining them from such sources.

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[1] From the experiments of Bouet (**2**) and Roubaud (**39**) there is evidence for regarding G. palpalis as the true host for T. dimorphon.

[2] In his most recent report Koch (**22**) states that in "Glossinen" (presumably G. palpalis) several species of trypanosomes can be found; one of which, so far found five times, can be identified with T. gambiense. It is not stated, however, in what way its identity is established; to judge from Koch's report, which is far from explicit, the identification was made on morphological grounds, a very unsafe criterion. Koch further states that in two of his five cases the trypanosomes were found in the salivary glands—a state of things contrary to all previous recorded experience (compare Stuhlmann [**41**], p. 23); without further explanation of these statements, further comment is impossible.

Another remarkable fact, to which I would draw special attention, is that trypanosomes, taken up into the digestive tract of the fly, do not infect susceptible hosts if artificially inoculated into them. Bruce ([**4**], p. 5) first discovered this curious fact, and could only infect with T. brucii from the stomachs of tsetses if inoculated not more than half an hour [1] after the fly had infected itself; all inoculations at later periods gave negative results. This has been confirmed by many subsequent investigators of trypanosome development, for example, by Prowazek (**36**), Koch ([**19**], p. 14), Bouet ([**2**], p. 474), Gray and Tulloch (**17**), and ourselves (pp. 227, 234). I agree with the explanation, first suggested I believe by Manson,[2] of this fact, that the trypanosomes in the digestive tract of the fly are developmental, and not merely multiplicative forms, and are not ripe, so to speak, for transference to the vertebrate host. Novy (**32**) considers this to be due to T. gambiense having died out in the flies, but this is certainly not so in our experiment (pp. 227, 234), in which case the fly's intestine contained numerous active T. gambiense. In the latter paper of Novy, MacNeal and Torrey (p. 262) it is stated that "the fact that ingested trypanosomes lose their virulence so rapidly in the stomachs of insects indicates a loss of functional activity, especially of the power of multiplication." This, again, is certainly not the case; multiplication of the trypanosomes proceeds continually in the gut of the insect.

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[1] Since infection by the bite of the fly can be obtained up to forty-eight hours, the trypanosomes inoculated by the tsetse must be those which remain in the proboscis and do not pass into the digestive tract. Bruce (**4**) observed trypanosomes of nagana in the proboscis as late as forty-six hours after feeding, though rarely. This agrees with the experimental results.

[2] I am not able to give the exact reference.

In my opinion the evidence now accumulated (I may mention especially Brumpt [**10**] and Prowazek [**36**] in addition to the authorities already cited) is conclusive in favour both of the general statement, that trypanosomes undergo development, as distinct from multiplication, in invertebrate hosts, and of the more special proposition, that certain species do so in tsetse-flies. I wish now to offer a few suggestions upon the mode of their development and the nature of the invertebrate cycle, as it may be termed. The possibilities of trypanosome development are bound up with the question of their possible or probable phylogeny and course of evolution in the past.[1]

Many authorities have pointed out that trypanosomes may possibly, if not certainly, have two distinct ancestral origins. The first is from a Trypanoplasma-like ancestor in which the anterior flagellum has been lost; this is the "trypanosome with flagellum morphologically posterior" of Léger (**24**). The second origin is from a Herpetomonas-like ancestor, in which the insertion of the flagellum becomes shifted backwards to form the undulating membrane; Léger's "trypanosome with the flagellum morphologically anterior." Woodcock (**42**) and Lühe (**26**) have gone so far as to base generic distinctions on this alleged difference of origin. For evidence bearing upon evolution in past times the zoologist turns naturally to the documents supplied by the records of development at the present time; and it must be acknowledged that so far there has been no development evidence whatever forthcoming in favour of a Trypanoplasma-like ancestor. No one has yet recorded a developmental stage of a trypanosome with two flagella, not even in those of fishes, which seem most likely to be allied to Trypanoplasma. On the other hand, Herpetomonas forms are common, if not invariable, in trypanosome development, and we may agree with Brumpt (**10**) and Léger (**24**) that in such cases this form certainly represents the ancestral form of these intestinal parasites before they became "sanguicolous." We may therefore look upon the Herpetomonas-form in the development of trypanosomes as a true recapitulative larval form, a type with which zoologists and embryologists are sufficiently familiar in the development of animals of all classes. For the present a Trypanoplasma-like ancestor most remain hypothetical until concrete evidence for it is forthcoming in given cases.

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[1] As I pointed out in the discussion on Hæmoflagellates before the British Medical Association at Exeter, where I have ventilated some of the views here expressed. My phylogenetic speculations were, however, put aside by Sir Patrick Manson, who expressed the view that where a parasite is now found in two hosts, for instance, a vertebrate and an arthropod, it was inherited by both of its hosts from their common ancestor (see 'Lancet,' September 7th,

Léger (**24**), confining his speculations to the trypano-

1907, p. 707). As I pointed out at the time, this view offers some difficulties; first, in view of the palæontological facts, showing vertebrates and arthropods to have been distinct in Silurian epochs, if not earlier; secondly, in view of the fact that "Hæmoprotozoa" divide their parasitism sometimes between an arthropod (insect or arachnid) and a vertebrate, sometimes between a leech and a vertebrate. I may point out further that the invertebrate host of a Hæmoprotozoon is always one that sucks the blood of the vertebrate; it remains, therefore, to be explained how the transmission from host to host was effected at the ancestral period when, ex hypothesi, all hosts of a given parasite belonged to a single species.

somes with flagellum morphologically anterior, considers that the Herpetomonas ancestor was "a primitively intestinal or entero-cœlomic[1] parasite of an invertebrate," not necessarily of a blood-sucking one, in which their entire life-cycle took place; that when the invertebrate became blood-sucking in habit, its gut parasites found themselves in a nutrient medium in which they were able to multiply enormously and were thus prepared for life in vertebrate blood, into which they finally succeeded in passing by inoculation through the proboscis. Their primitive habitat is supposed to be shown by the fact that only non-sexual multiplication takes place in the vertebrate, and the invertebrate host is necessary for their sexual development—a statement which, it may be remarked, is by no means definitely proved, however probable, for trypanosomes.

Léger's theory is logical and complete; it only seems to me to present one flaw. If we consider the transmission of trypanosomes generally, we find that it does not always take place by the intermediary of an insect, but may be effected by a leech; and if we include Hæmosporidia, as Léger does in his theory, we then have to reckon with Arachnida as well, in some cases. In other words, the constant trypanosome host is the vertebrate; the inconstant host is the invertebrate. I propose, therefore, to consider the facts from the standpoint of an opposite hypothesis, namely, that the ancestors of trypanosomes were primitively parasites of the gut of vertebrates, like so many flagellates known to exist at present, and that from the gut they passed into the blood of the vertebrate and finally into the gut of the blood-sucking invertebrate. If we attempt to imagine and to reconstruct on this basis the successive stages in the evolution of trypanosome life-cycles, we should probably have the following series of events:

(1) The ancestral form, ex hypothesi, was a flagellate parasitic in the vertebrate gut, which doubtless was

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[1] I am not quite sure that I understand the meaning of this adjective, though it has a familiar sound to the morphologist.

disseminated as such parasites are now; it formed resistent cysts in the gut which passed out with the fæces, were scattered abroad, and contaminated the food of fresh vertebrate hosts.

(2) When such a form succeeded in penetrating the intestinal wall and passing into the circulatory system, it found itself in a situation from which there was no escape or outlet by natural channels. Hence, if it did not at once come into relation with blood-sucking invertebrates, it could only have infected new hosts by coming back to the intestine of the vertebrate, becoming encysted there, and passing out with the fæces, as in (1). There is absolutely no evidence that any trypanosomes develop in this way; but a cycle of this kind has been described for Lankesterella ranarum by Hinze whose statements are usually, though perhaps not very logically, considered to be refuted by the observations of Siegel on the Hæmogregarina stepanowi of the tortoise.

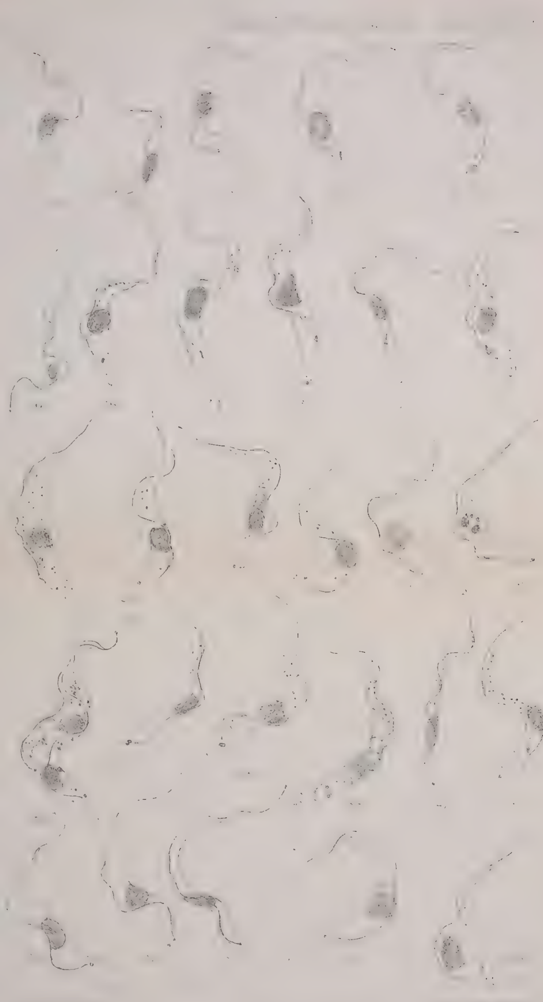
(3) Our trypanosome in the vertebrate blood may be supposed to have been taken up sooner or later by a blood-sucking invertebrate, the digestive juices of which it succeeded in resisting. It has acquired now a channel of escape from the vertebrate blood and is no longer obliged to become encysted in the vertebrate gut. Becoming adapted to the invertebrate gut, where it finds the nutriment, namely blood, to which it was accustomed, it now forms in the invertebrate gut the cysts which it formerly produced in the vertebrate. The cysts pass out with the fæces, are spread abroad, and reinfect the vertebrate host by contamination. This is the condition which I believe to be represented by T. grayi, described above; though it is possible that in this case contaminative infection is combined with inoculative, definite proof of either being as yet lacking.

(4) The trypanosome having become thoroughly adapted to the invertebrate gut acquires the power of passing forwards till it reaches its proboscis, and becomes inoculated into the vertebrate host, thus establishing the commonly-occurring inoculative type of infection. Now intestinal cysts become unnecessary and cease to be produced.

It is not necessary for me to dwell upon a possible fifth stage realised in Piroplasma, where the parasite passes through two generations of the invertebrate host. Nothing of the sort is known to occur in the case of trypanosomes.

It is not possible to prove or demonstrate a phylogenetic theory. One can only consider how far it suits known facts or overcomes difficulties. My theory has only one advantage over Léger's—that of explaining away the difference in the invertebrate hosts in different cases. Parasitic flagellates are found in the gut of invertebrates as well as vertebrates. If special stress be laid on the occurrence of Herpetomonas in insects which do not suck blood, such as the house-fly, I may refer to Prowazek's speculations on this form, especially his interesting suggestion that the house-fly is descended from blood-sucking ancestors, which acquired the Herpetomonas from vertebrate blood, so that H. muscæ-domesticæ would represent a stranded and persistent larval stage, comparable to, for instance, the axolotl amongst higher animals.

I have suggested above, and in a former memoir (**28**), the possibility that contaminative infection, the commonest of all methods of infection amongst Protozoan parasites generally, may occur also in the case of trypanosomes infecting vertebrates, basing my suggestion upon the encystation observed by me in T. grayi. Encystation has not been observed in any other species of trypanosome, and with regard to T. brucii, the observation of Stuhlmann (**41**) noted above, that the Herpetomonas-forms are found almost exclusively in the proboscis of the tsetse, rather indicates, as I have already pointed out, that the stage in the life-history which tends to become encysted in the case of T. grayi, does not do so in the case of T. brucii, which would probably be similar, in this respect, to T. gambiense. Moreover, experimental evidence, so far as it exists, is against the occurrence of contaminative infection; in the case of nagana Bruce (**4**) experimented with food and water from localities where the disease is rife, but failed to produce infection with it. Bruce also injected fæces of flies into susceptible animals,



TRYPANOSOMA GAMBIENSE

but without result; it is a pity the experiment was not tried of contaminating their food with the fæces. Hence the results to hand of observation and experiment, though they are very incomplete, indicate that the trypanosomes of the pathogenic group, such as T. brucii, belong to stage 4 of my hypothetical phylogeny. Nevertheless, if encystation occurs in one species of trypanosome it may occur in others.

Contaminative infection implies the infection of the vertebrate by way of the digestive tract. It is surprising how often the occurrence of accidental infections of this kind has been noted (see Laveran and Mesnil [**23**]) and yet how seldom it has been the object of direct experiment. Thus Bruce ([**8**], p. 46, Experiment 223) records the case of a dog which became infected with nagana after eating a piece of coagulated blood from the heart of a diseased heifer. It is usual to explain away such infections by supposing that an animal which becomes infected by way of the digestive tract must have had somewhere a lesion through which the trypanosomes penetrated, but this is pure assumption, and from the analogy of Dourine-infection it is quite as feasible to suppose that infection from the digestive tract can take place through the mucous membrane. If the trypanosomes can resist the digestive juices of insects, they may also resist those of vertebrates, and in this connection I may refer to the discovery by Léger (**25**) of an intestinal Trypanoplasma in the fish Box boops. All these facts seem to me to render perfectly possible and even probable the contaminative infection of the vertebrate, by way of the digestive tract, not merely as an exceptional occurrence, but as the normal course in those cases where, as in T. grayi, intestinal cysts are formed by the trypanosome. I may remark that if we start our phylogenetic deductions from Léger's hypothesis, it is not easy to explain the occurrence of encystation in T. grayi, unless we deny to it a vertebrate host altogether—a difficult position, it seems to me, in view of the habits of the fly.

The foregoing arguments may seem to many too speculative and unpractical. Phylogenetic speculations may, however, have a practical value, if only in widening our point of view, and at the same time formulating the possibilities of development, for which the investigator should be prepared. I think the studies of trypanosome development have been too much dominated by preconceived assumptions, and that investigators have too often been biassed by analogies and predisposed to force new facts into old formulæ. It is not necessary to suppose that the development of trypanosomes should be in all cases of the same pattern. If it be true that amongst trypanosomes two quite distinct lines of evolution are comprised, we may expect to find the greatest differences in their mode of development.

In conclusion, I may say that with regard to the main problem of my investigations, namely, the life-cycle of T. gambiense, it is a matter of great regret to me that I have little but negative results to bring forward, and can only offer speculations and hypotheses where I had hoped to have contributed definitely established facts. It was my desire to return to my investigations on this subject, but owing to the lack of the necessary support I have been obliged to abandon the idea. I think now, however, for reasons given above, it would be better to study the etiology of sleeping sickness in regions where it is endemic and where other species of tsetses besides G. palpalis occur. In both these respects Uganda is probably less suitable for the study of the problem than the Congo.

The following propositions summarise briefly the conclusions or personal opinions to which my investigations or reflections have led me.

(1) In Uganda Trypanosoma gambiense begins, but does not complete, a developmental cycle in Glossina palpalis, the method of transmission by this fly in this region being purely mechanical and direct.

(2) In other Diptera also, T. gambiense starts on a development in a precisely similar manner, but without getting so far, or persisting so long, as in Glossina palpalis.

(3) The observations and experiments of Koch, Stuhlmann, and others, show that T. brucii goes through a developmental cycle in G. fusca.

(4) It is probable that T. gambiense has an invertebrate host in which it completes its life-cycle in regions where it is indigenous, and it is possible that the true host may be Glossina fusca.

(5) Considerations of phylogeny indicate that the life-cycles of different trypanosomes should not be expected to be in all cases of one invariable type.

(6) The encystation observed in T. grayi indicates that contaminative infection may occur as well as inoculative.

V. Record of Observations.

The batches of flies used in the experiments here recorded were infected by feeding them once on an infected animal, in all cases a monkey. Those flies which did not feed on the infected animal were destroyed. The flies that had fed were kept alive by being re-fed on a "clean" animal, in almost all cases a guinea-pig, but sometimes a monkey. At first I used to feed the tsetses once every two days, but latterly I found it better to give the flies the chance of feeding daily. The method of feeding in all cases was as follows: The flies were kept in boxes with mosquito-netting at the sides and the box was placed on the skin (previously shaved) of the experimental animal, usually on the belly, so that the flies could feed through the gauze. As a rule they fed readily; indeed, care was necessary in handling the boxes to avoid getting one's fingers bitten through the gauze. The boxes containing the flies were kept over water, as without moisture the flies died quickly. The monkeys used in my experiments were species of Cercopithecus, either pygerythrus or smithi, and in all cases were infected, directly or indirectly, with trypanosomes from human cerebro-spinal fluid. The following is the record of the infected monkeys used by me, copied from the records of the Sleeping Sickness Commission, and kindly supplied to me by Gray, who entered them in the book.

"Monkey 404.

"June 7th, 1905.—Inject 5 c.c. of cerebro-spinal fluid from case of sleeping sickness, 'Sengoma.' Active trypanosomes are present in this fluid.

"July 16th.—Trypanosomes have not appeared in this animal's blood. Re-inject animal with a few drops of blood from Monkey 420. Trypanosomes are numerous in the blood of this latter animal (see Pl. VIII, figs. 22–25).

"Aug. 2nd.—Trypanosomes have appeared in this animal's blood to-day for the first time.

"Sept. 9th.—Trypanosomes have been regularly present in this animal's blood up to now. To-day the animal was sent to Nairobi at the request of the P. M. O. for the use of Dr. Ross, who is about to conduct some experiments on trypanosome transmission with the local tsetse-flies."

"Monkey 420.

"May 23rd, 1905.—Inject this monkey subcutaneously with 2 c.c. of cerebro-spinal fluid obtained from case of sleeping sickness, 'Vikitikeza.' Trypanosomes are numerous in this fluid.

"June 7th.—Trypanosomes have appeared in this animal's blood to-day for the first time. The parasites are scanty in numbers, and are long and thin for the most part.

"July 13th.—Trypanosomes are numerous in its blood.

"Sept. 18th.—The animal died to-day. Trypanosomes have been constantly present in its blood from the beginning. Post-mortem examination revealed nothing of interest."

"Monkey 478.

"Sept. 14th, 1905.—Inoculate this animal subcutaneously with 15 c.c. of cerebro-spinal fluid from a case of sleeping sickness, 'Sengoma.' Trypanosomes are numerous in the fluid.

"Sept. 26th.—Trypanosomes have appeared in this animal's blood to-day for the first time.

"Oct. 15th.—Trypanosomes numerous. Twelve flies fed on this animal to-day.

"Oct. 19th.—Trypanosomes numerous. Twenty flies fed to-day.

"Nov. 12th.—Trypanosomes numerous. Twenty flies fed to-day.

"Nov. 27th.—Died this morning. Trypanosomes numerous in the blood. One trypanosome seen in a smear of the brain. Glands and spleen somewhat enlarged. Stomach surface shows a few minute black ulcers and several nematode worms. Lungs, heart, etc., are normal."

"Monkey 507.

"Oct. 17th, 1905.—Inject the intestinal contents, with a few drops of normal citrate solution, of one Glossina palpalis subcutaneously into this monkey. This fly had fed on Monkey 478 some forty-six hours previously and was found on dissection to be swarming with trypanosomes (see p. 234).

"Dec. 5th.—This animal's blood has been regularly examined twice a week up to the present, but trypanosomes have never been found."

I begin the systematic record of my observations with the batch of July 31st, 1905. Previous to that date, however, I had made various tentative experiments and observations on the infection of the fly and on the changes of the trypanosomes in it, which I do not propose to record in detail.

Batch of July 31st, 1905.—The object of this experiment was to determine the changes undergone by T. gambiense in G. palpalis during the earliest periods of infection. A number of tsetse-flies freshly caught near Entebbe were fed on Monkey 420. Smears were made at the same time of the monkey's blood. Four of the flies were dissected one hour after feeding, and four more five hours after feeding.

The monkey's blood examined fresh showed trypanosomes fairly abundant, some more slender, active, others stouter, less active. In smears fixed with osmic vapour, well-marked differentiation of slender, stout, and intermediate forms were seen (see P. R. S., B 78, Pl. XII, figs. 16–19). A count gave 45 slender (8 dividing), 48 intermediate (8 dividing), and 3 stout forms.

In the smears from the flies dissected after one hour similar types of trypanosomes were seen, but the differentiation into slender and stout forms appeared to be more pronounced, and fewer intermediate forms were seen (figs. 36–39). The impression given by comparison of the monkey's blood with smears made from the fly at this stage is that the intermediate forms of the former have become converted for the most part into the stouter type of the latter. A count gave 37 slender forms (6 dividing), 13 intermediate forms (2 dividing), and 18 stout forms.

Smears from the flies dissected five hours after feeding showed the differentiation still further advanced, especially of the slender forms, some of which showed the characteristic clear cytoplasm free from granules. Stout forms were also found (figs. 40–44). A count gave 21 slender forms (3 dividing), 6 intermediate forms (1 dividing), and 11 stout forms (1 dividing).

Aug. 1st (twenty-four hours after infection).—Four flies were dissected, but smears were only made of two. The smears, both of the red and black blood, showed sharp differentiation into clear slender and granular stout forms (see P. R. S., B 78, Pl. XII, figs. 1, 2, 10, and 11). A count gave 45 slender (7 dividing), and 13 stout forms (1 dividing).

Aug. 2nd (forty-eight hours after infection).—Three flies were dissected and smears made of the blood. They showed trypanosomes of elongated, moderately stout form, and others of slender type. Dividing forms were also seen (P. R. S., B 78, Pl. XII, fig. 12).

A remarkable feature of this batch is the excess of slender forms over stout.

Batch of Sept. 8th, 1905.—A batch of flies, caught on the island of Kimmi, were fed on Monkey 420. Four flies were dissected after two hours, three after six hours, and three after twelve hours.

The smears made from the flies dissected after two hours showed slender, stout, and intermediate forms, with many division stages.

In the smears made from flies six hours after feeding, the differentiation of stout and slender forms was more marked. One fly contained Trypanosoma grayi as well as T. gambiense; unfortunately the preparations were a failure.

At twelve hours after feeding the differentiation of the stout and slender forms appeared to be complete.

Sept. 9th (twenty-four hours after infection).—Three flies were dissected and smears made from them. A fourth fly was dissected and the blood examined fresh. In the latter no trypanosomes were seen in the red blood; in the black blood they were found singly and in groups of two or three or even four. The single ones moved rapidly, with flagellum forwards. In the groups they were attached by the posterior ends with the flagella free; individuals thus attached were sometimes unequal in size. A group of three was observed in which four flagella could be distinctly seen, indicating division of one of them. A group of four was noted in which one was much smaller than the others, and one larger; this group separated into two couples; in each couple the trypanosomes were attached tête bêche, the two *nn* opposite and close together (fig. 110). The smears showed trypanosomes differentiated into the normal slender, clear, and stout, granular forms (P. R. S., B 78, Pl. XII, figs. 3–5 and 7–9). Dividing stages of both forms were common.

Sept. 10th (forty-eight hours after infection).—Three flies were dissected; the most successful preparation showed long, moderately stout or slender forms (see P. R. S., Pl. XII, fig. 15); the slender clear forms of the previous day wanting.

Sept. 12th (ninety-six hours after infection).—Three flies were dissected, and smears made of the blood. In the first two flies no trypanosomes were found; in smears of the third trypanosomes were found scantily in the red blood, but fairly abundant in the black. They were, for the most part, remarkable for their large size (p. 183, and Pl. X, figs. 97–101).

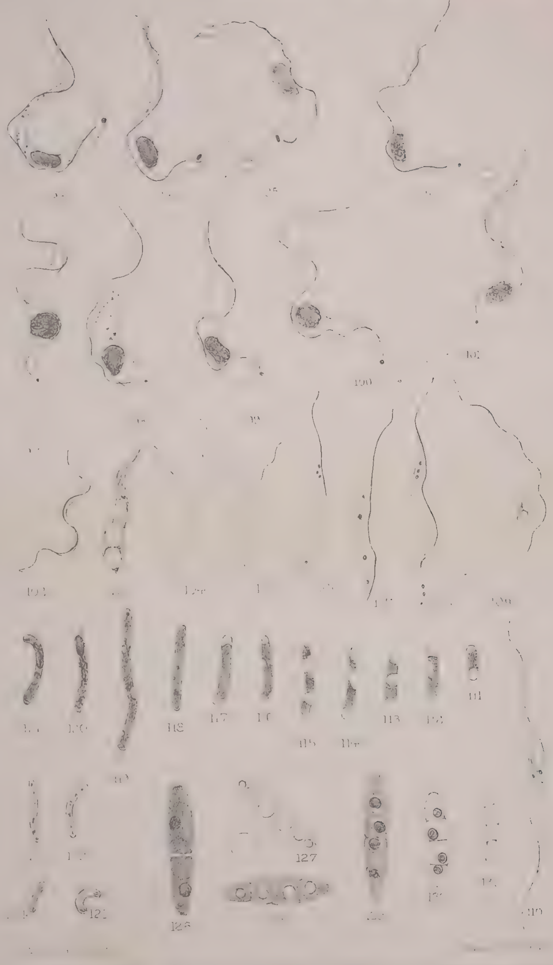
Batch of Oct. 1st, 1905.—This batch consisted of ten flies bred from pupæ. They were put on to Monkey 478, which was showing a fair number of T. gambiense in its blood, some smears being made from the monkey at the same time. Seven of the flies fed, three refused.

The smears of the monkey's blood showed trypanosomes chiefly of an indifferent type (figs. 1–5). None of the typical stumpy forms were found, but after some searching a few slender forms (fig. 5) were found. In all 83 indifferent forms, 10 slender, and 1 dividing form were counted in a smear.

Of the seven flies that fed one died before being examined; the remaining six were dissected on Oct. 2nd, 3rd, 4th, 5th, 6th, and 8th respectively. All their organs were examined very carefully, namely the pericardial fluid, Malpighian tubules, salivary glands, proventriculus, stomach, intestine, proctodæum, and in some cases the genitalia. In no case were trypanosomes found of any sort. Some of the flies were full of bacteria.

The negative result obtained in this batch was remarkable and difficult to explain. Two points call for notice—the very slight amount of differentiation in the trypanosomes of the monkey's blood, and the fact that the flies were bred in captivity and probably rather sickly and delicate; but whether the result is to be explained by either of these data cannot be asserted definitely.

Batch of Oct. 3rd.—This consisted of a few flies caught the day previously in the vicinity of Entebbe, and fed on



TRYPANOSOMA GAMBIENSE: [illegible] BACTERIA [illegible]

Monkey 478. The flies were kept in a large cage, with water and vegetation, in a situation where direct sunlight fell on the cage late in the afternoon. Smears of the monkey's blood made the day before (Oct. 2nd) showed a considerable amount of differentiation (figs. 6–10), in contrast with the smears made from the monkey the day previously (Oct. 1st). Stout, slender, and indifferent types could be distinguished; of the first 99, of the second 97, and of the third 34 were counted in one smear. Most of the stouter forms were of the typical stumpy form (figs. 9 and 10). Specially noteworthy is the length of the free flagellum in slender forms, and its shortness in the stout forms (compare figs. 6 and 7); by this character the indifferent forms could also be subdivided into two classes.

It is interesting that the trypanosomes from the blood of the same monkey on two consecutive days should show so little differentiation on one day, so much the next day.

Oct. 4th (twenty-four hours after infection).—Two flies were dissected. In the first a few trypanosomes of the stout type were seen in the black blood. A smear was made in which seven typical stout trypanosomes were counted. In the second fly no trypanosomes were found; the stomach contained a vast number of bacteria.

Oct. 5th (forty-eight hours after infection).—One fly was dissected, and various organs examined. Trypanosomes were found fairly abundantly in the red blood and the black blood. The sucking stomach contained a peculiar clot of blood, in which, however, no trypanosomes were found. The trypanosomes in the fresh condition appeared chiefly of the broad type, but one of more serpentine appearance with pointed posterior end was noted.

The smears made were unfortunately very unsatisfactory as regards preservation. In the best one, of the red blood, a few trypanosomes of moderately stout or moderately slender type (figs. 66–68) were found; in some parts of the smear they occurred in clumps, three or four together. In the smears of the black blood none were found.

Oct. 6th (seventy-two hours).—One fly was dissected; there was no blood in the digestive tract, only some brownish fluid in the intestine, in which two trypanosomes were seen. One smear was made, but no trypanosomes could be found in it.

Batch of Oct. 9th, 1905.—This batch consisted of two flies, both of which had been bred from pupæ in August, and had been kept since then in a fly-cage and fed regularly every two or three days on a fowl. The same fowl had been used for feeding the flies in the breeding-cage—that is to say, flies caught wild near Entebbe and kept in a large cage in order to obtain pupæ from them. The fowl became emaciated and of sickly appearance and died; its place was then taken by a second fowl. Unfortunately the fowl that died was not examined.

On Oct. 9th the two flies were fed on Monkey 409, which was showing a fair number of trypanosomes (T. gambiense) in its blood.

Oct. 10th (twenty-one hours after infection). — One of the flies were dissected. Both stomach and intestine were full of red and black blood respectively, and both were swarming with Trypanosoma grayi. In the proctodæum a few non-motile slender trypanosomes, apparently dead, were seen. No trypanosomes were found in the pericardial fluid, salivary glands, proventriculus, thoracic intestine, or sucking stomach. Examination of smears gave the following results: In all T. grayi was abundant, but prolonged searching was necessary to find T. gambiense, a specimen of which, from this fly, was figured by me (P. R. S., B 78, Pl. XII, fig. 14).

The smears of the red blood showed great variety of forms. The following types could be distinguished: (1) large forms, with hinder end narrow (figs. 218, 219); (2) large forms, with hinder end swollen, apparently about to divide (fig. 221); (3) quite small forms (fig. 220); (4) medium-sized, apparently representing stages in the growth of (3) into (1) and (2); (5) round forms, connected by transitions with (3) (figs. 222, 223); (6) slender forms (figs. 224–226); (7) dividing forms. A count gave the following numbers of each type:

	(1)	(2)	(3)	(4)	(5)	(6)	(7)
Numbers .	10	34	7	5	19	26	2
Percentage.	9·7	33	6·79	4·85	18·44	25·24	1·94

The smears of the black blood showed the same types, (1) being perhaps rather more pronounced in its characteristics (figs. 226 *a*–231). A count gave the following results:

	(1)	(2)	(3)	(4)	(5)	(6)	(7)
Numbers .	86	31	23	22	3	60	13
Percentage.	36·38	13	9·66	9·24	1·26	25·25	5·46

The two smears made from the proctodæum gave remarkable results. In one after much searching a single, slender form was found. In the other, vast swarms of trypanosomes were found in certain spots; in particular, one huge clump, quite visible to the naked eye, and resembling under a low power a section of spleen or other small-celled tissue. Away from these clumps trypanosomes were scanty. The majority of the trypanosomes were slender, Herpetomonas-like forms (fig. 232), but, unfortunately, badly preserved, having a macerated appearance, with *n* often broken up. A few stouter forms (fig. 233), and some pear-shaped forms without flagellum were also to be found. The fact that very scanty free trypanosomes were seen in the fresh condition indicates that the individuals found in the smears were probably all attached in clumps to the wall of the proctodæum, as noted in the fly of Nov. 14th (p. 245). The fact that the few free trypanosomes seen were non-motile, and that in the smear they appear macerated, indicates that the trypanosomes were moribund for some reason. (I have seen similar results follow from using a dissecting needle, which had been inadvertently dipped into borax carmine.)

Oct. 11th (forty-five hours after infection). The second fly of the batch was dissected; it was found to be very anæmic, and its digestive tract contained no blood, but swarms of large bacteria. No trypanosomes were found.

Batch of Oct. 15th.—A number of flies, freshly caught near Entebbe, were fed upon Monkey 478.

Oct. 16th.—Four flies (one female, three males) were dissected, at about twenty-one, twenty-four, twenty-seven, and twenty-eight hours after infection respectively. In all of them trypanosomes were found both in the red and black blood, not very abundantly, but slightly more so in the black blood. They were for the most part of stout form, but some slender ones were seen also. In the black blood from the second fly two trypanosomes were seen adhering together by the hinder end; after a time a third added itself to them.

Smears were made from all the flies, both of red and black blood; they showed trypanosomes rather scanty in number, but sharply differentiated into slender and stout types (figs. 45–47); very few dividing forms were seen. In one smear, 33 stout and 4 slender were counted; in another, 88 stout, 4 slender, and 3 dividing.

Oct. 17th.—Five flies were dissected. The first fly (male, forty-five hours after infection) was plump and fairly full of blood, both red and black. Trypanosomes were seen fairly abundant in the red, still more so in the black blood; in the latter many of them were united by the posterior end into groups of as many as five. As these trypanosomes appeared active and healthy, as well as numerous, the experiment was made of injecting some of both red and black blood into a monkey (No. 509), but the injection was without effect, and the monkey never became infected (see p. 227).

Smears of the red blood showed trypanosomes sharply differentiated into stout and slender forms (figs. 76, 77), similar to those found the day previous. Smears of the black blood, however, showed a slightly different type, moderately stout, rather long, and characterised, for the most part, by considerable length of the free flagellum (figs. 73–75).

The second fly had the gut perfectly empty of blood, and contained no trypanosomes.

The third fly (female, forty-seven hours after infection) was gorged with blood. The trypanosomes were not very numerous; some were more slender, others stouter in form; in the red blood a slender one and a stout one were seen hanging on together.

Smears of the red blood showed stout forms, not very numerous, one of which was dividing (fig. 69). Smears of the black blood showed trypanosomes fairly numerous, varying from a moderately slender to a very stout type (figs. 70–72). In one smear were counted 17 slender, 25 stout, 21 intermediate, and 2 dividing forms.

The fourth fly (male, forty-eight hours after infection) was of emaciated appearance; the stomach was full of bacteria and contained no red blood; the intestine contained black blood in which were a few motile trypanosomes. Two smears were made, in each of which a single trypanosome of stout form was found.

The fifth fly (male, fifty hours after infection) had a small amount of red blood, in which no trypanosomes were seen, and a considerable amount of black blood, in which a few active trypanosomes were seen. In the smears, however, no trypanosomes could be found.

Oct. 18th.—Three flies dissected and examined. The first fly (male, sixty-nine hours after infection, not re-fed) showed a small amount of blood in the intestine, black posteriorly, reddish anteriorly. A few motile trypanosomes were seen; none in the proventriculus. In a smear a fair number of trypanosomes were seen, slender or moderately stout in type, some of them very granular; unfortunately the stain was very faint.

The second fly (male, seventy hours after infection, not re-fed) showed a similar condition to the last. A fair number of active trypanosomes were seen in the intestine, some of them long and moderately slender, others broader. Three trypanosomes, originally quite separate, were seen to come together and adhere by their posterior ends. No trypanosomes were found in the proventriculus, proctodæum, or sucking stomach. The two smears made showed trypanosomes for the most part long and moderately stout (fig. 87), others more slender (fig. 86), and others again stouter (fig. 88), all of them very granular and very different in form and appearance from the two types found at twenty-four hours.

The third fly (female, seventy-two hours, re-fed at fifty-one hours after infection) had the digestive tract gorged with blood, red in the stomach, black in the intestine. No trypanosomes were found in the red blood, but in the black blood as many as three or four in a field (3 mm. apochr. immersion); at first very active, they soon became sluggish under observation. A stout one was seen with very distinct undulating membrane, and with *n* and *N* apparently dividing (fig. 109). Another rather smaller one had the undulating membrane distinct, and the cytoplasm containing refringent granules (fig. 107). A third was long and slender, with groups of refringent granules (fig. 108). Of the red blood five smears were made, and, after much searching, two trypanosomes were found in one of them; one of these was damaged, the other (fig. 82 *a*) was of extremely large size, very granular and vacuolated, and in process of division.

My two smears of the black blood show fairly numerous trypanosomes (figs. 83–85), most of them remarkable for their length and large size, varying in type from slender to stout; they are nearly always very granular and frequently vacuolated. Dividing forms were also seen.

Oct. 19th (ninety-four to ninety-eight hours after infection).—Three flies dissected, two male and one female; the two first had been re-fed Oct. 17th, the last re-fed Oct. 18th. All three flies appeared healthy and full of blood, red and black, but no trypanosomes were found in any of them. Numerous smears were made of each and carefully searched, but no trypanosomes could be found.

Oct. 20th (118–119 hours after infection).—The last two remaining flies (both males) were dissected and examined. No trypanosomes could be found in the digestive tract, neither in the fresh condition nor in smears.

Batch of Oct. 19th, 1905.—A number of freshly-caught Entebbe flies were fed on Monkey 478. At the same time smears were made of the monkey's blood, which showed trypanosomes rather scanty but comprising typical long, slender, and short, stumpy forms, as well as others of more indifferent type (figs. 11–13).

Oct. 20th (twenty-four hours after infection).—One fly dissected. The digestive tract was gorged with blood. Trypanosomes were very scanty in the red blood, more abundant in the black. Smears were made of both, and showed the typical slender and stout forms well differentiated, but rather scanty in number; the stout forms rather in excess.

Oct. 21st (forty-six to fifty hours after infection).—Four flies were dissected (three males, one female). In all of them only black blood was found; in one no trypanosomes were seen, but in the other three flies active trypanosomes of moderately slender type were noted. Smears made showed trypanosomes present, but scanty in number, in all four flies. The trypanosomes were long, varying from moderately slender to moderately stout in type, usually very granular, sometimes vacuolated (figs. 78–80). Dividing forms were seen.

Oct. 22nd.—Three flies were dissected. The first fly (female, seventy hours after infection; re-fed Oct. 21st) had the stomach and intestine very full of red and black blood respectively; the black blood contained a few crystals. Trypanosomes were fairly numerous in the black blood and of slender, active type, similar to T. grayi. One that was watched moved rapidly "en flêche" across the field, and was difficult to keep in view. None were seen in the red blood or in the proventriculus. Smears were made both of red and black blood; in the former no trypanosomes were found. In the smears of the black blood the trypanosomes were not very numerous, but very uniform in type (figs. 93–96). They were all of considerable length, moderately stout or moderately slender. (Their slender appearance in life is perhaps to be taken in a proportionate sense—that is to say, it was their length which gave the impression of slenderness). Their cytoplasm was not granular or vacuolated, and they appeared perfectly healthy and normal; the free flagellum was relatively short; *n* relatively large, sometimes rod-shaped. No dividing forms were seen.

The second fly (male, seventy-one hours after infection, re-fed Oct. 21st) showed exactly the same condition as the last, and in this case also no trypanosomes were found in the red blood, but in the black blood they were numerous; some more slender in appearance and active in movement, progressing rapidly en flêche; others stouter, slower in movement. Smears were made of the black blood; unfortunately the preparations were not very successful, but they showed trypanosomes similar to those seen in the first fly (figs. 89–92), some very slender.

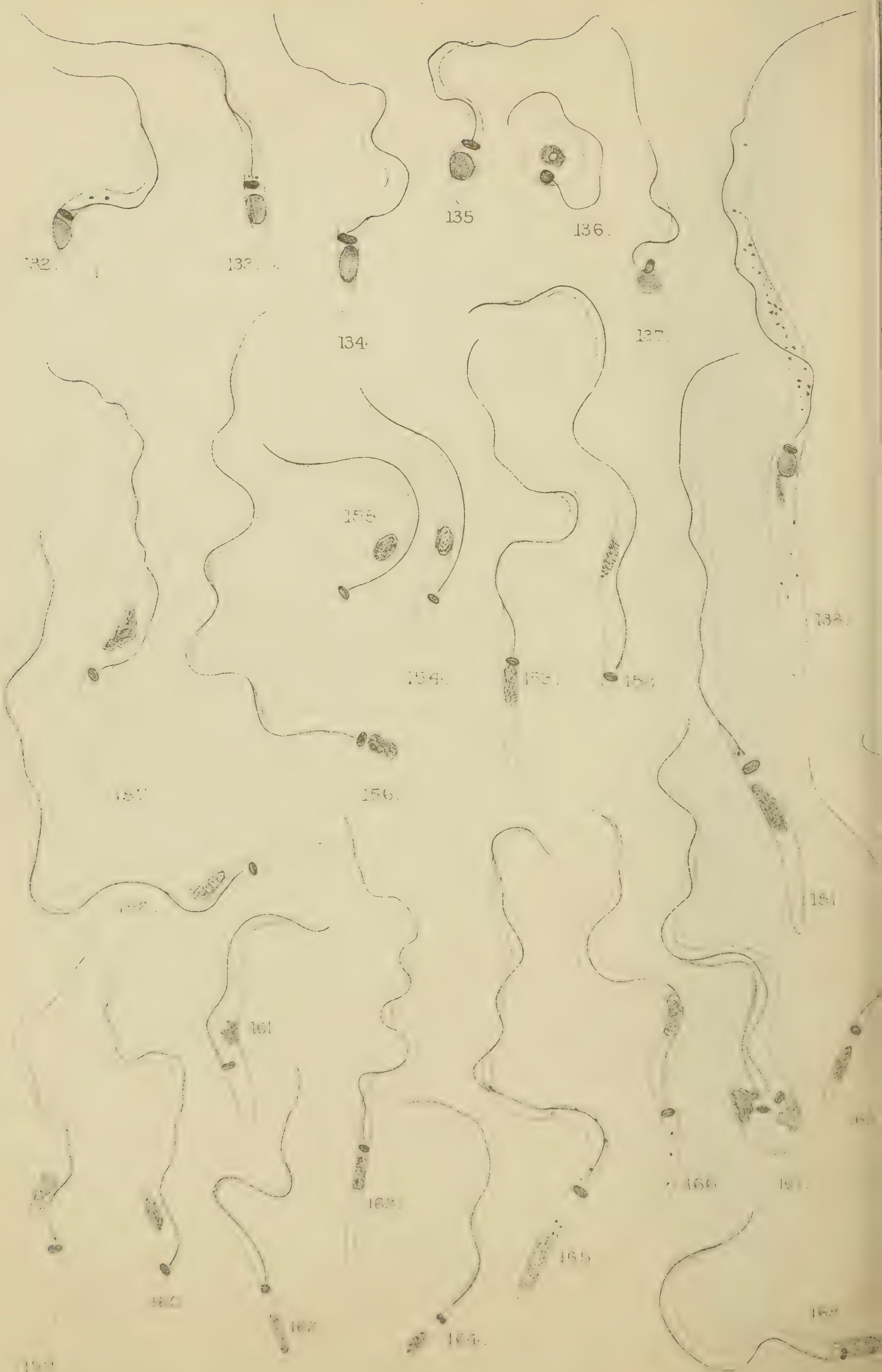
The third fly (female, seventy-two hours after infection, re-fed Oct. 21st) showed the same condition as the two already mentioned. In the black blood a few trypanosomes were seen, both slender and stout, but less active than those seen in the other two flies. The smears made were defective, but in one of them two trypanosomes of rather stout build were seen.

Oct. 23rd (ninety-four to ninety-eight hours after infection, re-fed Oct. 21st). The five remaining flies were dissected; in all of them both red and black blood was present in the digestive tract; the black blood contained numerous crystals. No trypanosomes were found in the red or black blood, nor in the proventriculus nor proctodæum. Smears were made of the blood, but prolonged searching failed to reveal any trypanosomes.

The remaining eleven flies of this batch were examined at intervals up to Nov. 1st, on which date the last seven were examined; in no case were any trypanosomes found.

Batch of Nov. 1st, 1905.—Some freshly-caught Entebbe flies were fed on Monkey 405, which, however, was showing very few trypanosomes in its blood.

Nov. 2nd.—Some of the flies were dissected; no trypanosomes were found in them with the exception of one, which



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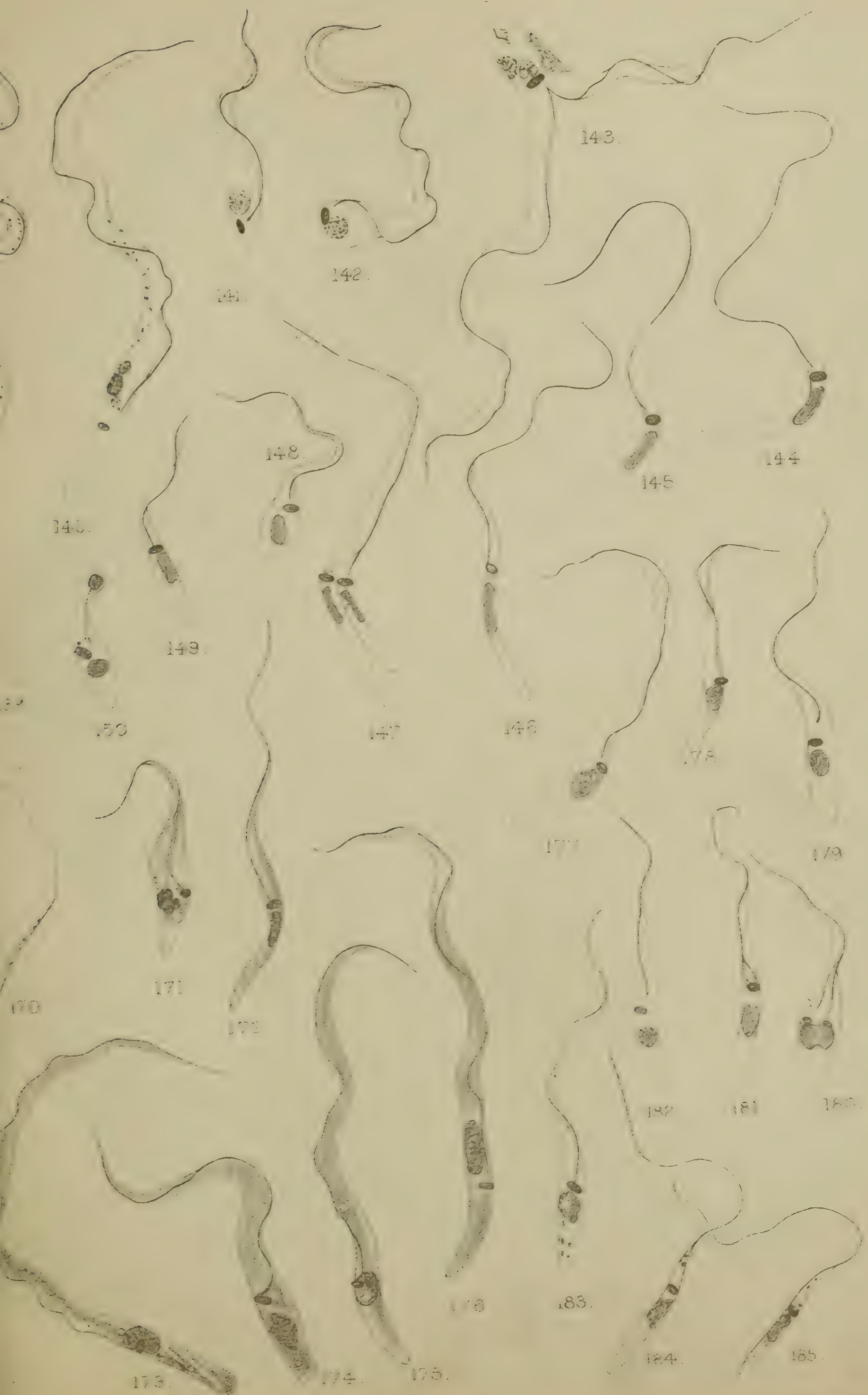

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was found swarming with T. grayi. Five smears were made, but unfortunately no note was kept as to the parts of the gut from which the smears came—an oversight much to be regretted, as the preparations show trypanosomes of a remarkable type; slender ones varying from small (figs. 235, 236) and medium-sized (fig. 241) to a very great length (figs. 242–244). In some of the very long ones *n* could not be made out clearly, perhaps on account of defective preservation; in others *n* distinct. Large forms also occur (fig. 238), and a form with *N* posterior (fig. 234) is also common. In nearly every case *n* is well in front of *N*; only occasionally *n* at the side, and in two cases noted to be behind *N*. A few dividing forms were seen. In the preparations fixed with absolute alcohol the trypanosomes frequently appeared very granular, and full of chromidia.

Batch of Nov. 3rd, 1905.—A batch of Glossina palpalis, freshly caught, was fed on Monkey 478 (see p. 227), which was showing a fair number of trypanosomes in its blood. Smears were made at the same time of the blood of the monkey. The batch of flies was re-fed on healthy animals on the 5th, 7th, and 8th, and after that daily, until the flies had all been used up for dissection and examination.

The smears of the monkey's blood showed trypanosomes fairly abundant and of various types (see figs. 14–17). Of 212 trypanosomes counted, it is noteworthy that only one was dividing; the remainder comprised the following types of form: stumpy forms (figs. 16, 17), 82 (= 38·1 per cent.); long forms (fig. 14), 63 (= 31·2 per cent.); intermediate forms, slender, 28 (= 12·6 per cent.); intermediate, broad (fig. 15), 38 (= 17·5 per cent.). This shows a slight preponderance of stout over slender types, roughly 4 : 3.

Nov. 4th (twenty-four hours approximately after infection). —Three flies were dissected and examined, and smears made from each. In all of them active trypanosomes were found easily in the black blood, when examined fresh, but a hasty examination of the red blood did not reveal any trypanosomes;

they were found, however, in the smears of both red and black blood. The trypanosomes showed pronounced differentiation into slender and stout types (figs. 48–50), the latter greatly preponderating in number. A small number were seen to be dividing, in nearly all cases stout forms. Examination of seven slides gave the following results :

	Slender.	Stout.	Dividing.
1st fly, red blood	0	11	0
1st fly, red blood	15	64	1
1st fly, black blood	0	23	0
2nd fly, red blood	1	29	0
2nd fly, black blood	10	33	6
3rd fly, red blood	2	7	0
3rd fly, black blood	9	269	4
Total	37	436	11

This gives roughly twelve stout to one slender form.

Nov. 6th (seventy-two hours after infection).—One fly was dissected; the red blood examined fresh showed no trypanosomes, but in the black blood some active trypanosomes were found, chiefly of slender type; one large stout form, however, was seen. Three smears of the black blood were made, but put aside and fixed and stained in England; only on one of them were two trypanosomes found after much searching; both, unfortunately, were badly preserved.

Nov. 7th (ninety-six hours after infection).—Three flies were dissected and all their organs carefully examined for trypanosomes. The flies were apparently healthy and contained both red and black blood. In the first fly (male) a few scarce trypanosomes were noted (by my colleague F. M. Tulloch) in both the red and black blood, but in four smears made (two of each kind of blood) none could be found after prolonged searching. In the second fly (female) one active trypanosome was seen in the black blood, after much searching, but in the smear that was made none could be found. In the third fly (female) no trypanosomes were found.

Nov. 8th (120 hours after infection).—Five flies (four males, one female) were dissected and examined for trypanosomes. In each case the examination was extended to the red and black blood, the proventriculus and proctodæum, and either the testes and seminal vesicles, or the ovaries and the larva (present in one case). No trypanosomes were found.

Nov. 10th (168 hours after infection).—Three flies (all males) were dissected and all organs carefully examined. In one of them nothing was found; the other two were found to be infected with Trypanosoma grayi.

In the first fly the intestine contained a small amount of black blood, in which were corpuscles of normal appearance. No trypanosomes were found in the proventriculus, black blood, testes, or seminal vesicles, but the proctodæum contained slender, very active, and rapidly motile trypanosomes. One smear was made which showed fairly numerous trypanosomes of moderately slender type (figs. 132–135). Round forms (fig. 136) also occur, and others transitional (fig. 137), between the round forms and the prevailing slender type. No dividing forms were seen, nor any encysting forms (for further description see p. 195).

In the second fly also the intestine contained a fair amount of black blood and no red. Trypanosomes were found swarming in the proctodæum and black blood; none were found in the proventriculus, the testes, or the seminal vesicles. Smears were made of the proctodæum and the black blood. The smears of the proctodæum showed an immense number of slender forms with very long flagella, *n* large, transverse, always in front of *N* (figs. 144–146), also a few plumper, shorter forms, with the flagellum short and thick, as if being retracted, as in encysting forms, but without any cyst-wall (figs. 148–150); and, finally, a very few cysts, which, however, always showed the cyst-wall damaged and spread out, probably owing to the thinness of the smear; no dividing forms seen, with one doubtful exception (fig. 147). In the smears of the black blood trypanosomes were also found very abundantly and of types startlingly different from those in the proctodæum. The trypanosomes exhibited great variation of form, size, and structure, but could be divided into four types: (1) The prevailing type was a large vermiform trypanosome with a very short, free flagellum, the anterior end blunt, and the posterior end narrow and drawn out (figs. 138–139); (2) others had the anterior end more tapering, the posterior more or less swollen (fig. 140); (3) a certain number were broad and fat, as if about to multiply by division (fig. 142); in addition to these three types there were found (4) many small or medium sized, as if resulting from recent division (fig. 141); on the other hand, division stages were extremely rare. In all these forms from the black blood the relative positions of *N* and *N* were extremely variable (for further details see p. 190). A count made from the two best preparations gave the following numbers for the various types:

	1. Vermiform.	2. Tadpole-like.	3. Large, fat.	4. Young, small.	5. Young, medium-sized.	6. Dividing.
1st preparation .	80	20	14	65	34	0
2nd preparation .	197	152		101	34	5
Total . . .	277	186		166	68	5
Percentage .	38·6	26·49		23·64	9·67	0·71

Nov. 11th (192 hours after infection).—One fly (male) was dissected; the intestine contained a small quantity of black blood; the stomach contained no blood but quantities of bacteria. No trypanosomes were found in the black blood, proctodæum, stomach, proventriculus, sucking stomach, testes, or seminal vesicles.

Nov. 12th (216 hours after infection).—Three flies (all males) were dissected; two of them contained red and black blood, one only black; in all three the stomach, intestine, proventriculus proctodæum, and genitalia were examined; no trypanosomes were found.

Nov. 13th (240 hours after infection).—Three flies were dissected. The first fly (male) was gorged with blood, both red and black; no trypanosomes were found in the red or black blood, the proctodæum, salivary glands, or genitalia. The second fly (male) had the intestine empty except for a small quantity of blackish fluid in the intestine; the stomach was crammed with bacteria; no trypanosomes were found in the stomach, intestine, proctodæum, proventriculus, salivary glands, or genitalia.

The third fly (female) had a small quantity of red blood in the stomach, and the intestine full of black blood; it was found to be swarming with Trypanosoma grayi throughout the stomach, intestine, and proctodæum, but no trypanosomes were found in the salivary glands, larva, or proventriculus; one was found, however, in a teased-up ovary, but this was probably accidental; these excessively motile parasites are often let free during dissection by accidental ruptures of the gut-wall, and swarm out into the salt solution in which the dissection is performed.

Examination of the smears made from this fly gave the following results: In the red blood (figs. 151–155) trypanosomes were excessively abundant, for the most part of moderate size, fairly slender, the free flagellum often very short, sometimes long, and the relative positions of *N* and *n* extraordinarily variable (see p. 190). A count gave the following numbers of different types:

	Large, slender.	Large, stout.	Small.	Medium-sized.	Dividing.
Numbers .	240	12	157	41	9
Percentage .	52·28	2·6	34·2	8·93	1·96

In the black blood also the trypanosomes were very abundant and in the main similar in type to those in the red blood, perhaps rather more slender, more active in appearance (figs. 156–162); but in addition there occurred a small number of slender Herpetomonas-like forms, distinguished at once by their very slender form and transparent appearance; they stain feebly, and also have *n* in front of *N* (figs.

163, 164). A count gave the following numbers of the different types:

	Large, slender.	Large, stout.	Small.	Medium-sized.	Slender, H-like.	Dividing.
1st smear . .	248	90	27	27	103	9
2nd smear . .	258	24	37	13	94	8
Total. . .	506	114	64	40	197	17
Percentage .	53·94	12·15	6·82	4·26	21	1·81

Of the contents of the proctodæum I have two smears; in one, which I may call the pure smear, there are no blood-corpuscles; in the other there is a slight admixture of black blood and the preparation contains blood-corpuscles. The trypanosomes show the same types as in the black blood (figs. 165–170), and a count of the pure smear gave the following results:

	Large, slender.	Large, stout.	Small.	Medium-sized.	Slender, H-like.	Dividing.
Numbers . .	258	62	21	4	106	24
Percentage . .	54·31	13	4·42	0·84	22·31	5·05

Comparison of the figures shows that the percentage of Herpetomonas-like forms is about the same in the black blood and the proctodæum.

Nov. 14th (264 hours after infection).—Three flies (all males) were dissected. In two of them the stomach contained no red blood, but numerous bacteria, and the intestine contained blackish fluid; in both of these the proventriculus, stomach, intestine, protodæum, testes, and seminal vesicles, and in one the salivary glands, were examined without finding any trypanosomes.

In the third fly the digestive tract was found gorged with blood, both red in the stomach and black in the intestine. Trypanosoma grayi was found present in stupendous numbers throughout the stomach, intestine, and proctodæum; in the last named the trypanosomes were found both free and swimming actively and also adherent to the wall in large patches, resembling, under the low power of the microscope, patches of mould, the whole mass quivering and vibrating with the movements of the flagella. No trypanosomes were found in the testes, seminal vesicles, salivary glands, or proventriculus.

Examination of smears gave the following results: The red blood showed a preponderance of large forms (fig. 173), together with a certain number of small or medium-sized trypanosomes (fig. 171) and a few division stages; slender forms (fig. 172) were also found, but were very scarce. A count gave the following results.

	Large.	Medium.	Small.	Male.	Dividing.
Numbers .	237	64	63	3	14
Percentage .	62·2	16·79	16·5	0·78	3·6

The transitional region between the red and black blood showed a similar state of things, but the trypanosomes were rather smaller, not attaining such great length as in the red blood (figs. 174–176). Division stages were frequent, and a series has been drawn from the two smears of this region (figs. 204–216). No slender forms were found.

The black blood (figs. 177–182) showed in the smears a few large forms, and some of medium size (fig. 177), but the great majority were small forms, like those recently originated from division. In one slide forms were found, which, by their shape—especially the pointed hinder extremity (fig. 178)—approached the Herpetomonas type, but true H-forms were not found. A few round forms also were found. A count resulted as follows:

	Large or medium.	Small.	Round.
Numbers	162	749	23
Percentage	17·34	80·1	2·46

Thus over 80 per cent. were small forms, all of them with *n* in front of *N*, or at the side; none with *n* behind *N*. In the larger forms the relative position of *n* and *N* varies greatly (see p. 190). Dividing stages were very rare; one is figured (fig. 180).

The smears[1] from the proctodæum showed free forms and all stages of encystment. The free forms show two types: (1) larger, broader forms, resembling the small forms seen in the black blood (figs. 183, 186); (2) slender, Herpetomonas-like forms with narrow bodies pointed posteriorly, and very long flagella, faintly stained (figs. 184, 185, 187, 188). The encysting forms, though forming a continuous series, may be divided into (3) forms with flagellum recognisable (fig. 189–193); (4) pear-shaped with flagellum retracted (figs. 194–198); (5) ripe cysts, more or less spherical (figs. 199–202). A count gave the following results:

	1.	2.	3.	4.	5.	6.	7.
	Ordinary.	H-like.	Encysting flagellum present.	Flagellum retracted.	Ripe cysts.	Round forms.	Dividing forms.
Number .	41[2]	60	38	62	30	2	1
Percentage.	17·48	25·58	16·22	26·42	12·82	·84	·42

Nov. 15th (288 hours after infection).—Two flies, the last survivors of the batch, were dissected, one a male, the other a female, containing a larva. Both were gorged with blood, red in the stomach, black in the intestine, of both the proventriculus, stomach, intestine, proctodæum, genitalia, and in the female the larva were examined, but no trypanosomes were found.

Stomoxys Experiments.

Nov. 17th.—A batch of Stomoxys sp. fed on Monkey 510, which was showing a fair number of trypanosomes.

Nov. 18th.—Four flies dissected and examined; in all try-

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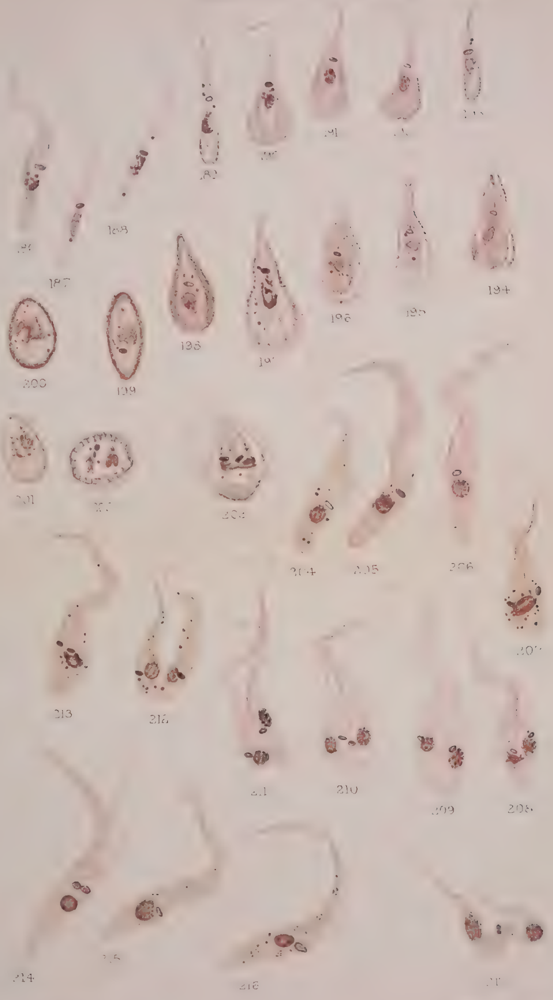
[1] Although the proctodæum contained no blood, my smears show a few blood-corpuscles. I think these must have come from the serum used in making the preparations.

[2] One, and only one, of this number had *n* behind *N*.



[illegible]

TRYPANOSOMA GRAYI (Figs. 186–216). T. GAMBIENSE (Fig. 217).

panosomes were found in the fresh state. Smears were made and put by for examination. In the smears of two flies no trypanosomes were found; in a third fly they were found in the smears of red blood, not in the black; in the fourth fly they were found both in the red and the black blood. The trypanosomes were slender and stout forms perfectly typical in character (figs. 51–55), the former scarce, and some of the latter dividing. In a smear from one fly 66 stout (6 dividing) were counted, but no slender; in a smear from the other fly 28 stout, of which 11 were dividing, and 4 slender were counted. The high proportion of division-stages is remarkable.

Nov. 19th.—The remaining Stomoxys, 20 in all, were dissected and examined, but no trypanosomes were found in any of them.

Mansonia Experiments.

Nov. 19th.—Two mosquitoes (Mansonia sp.) were fed on Monkey 478.

Nov. 20th.—One was dissected and somewhat hastily examined without any trypanosomes being noted. In the smears, however, trypanosomes were found abundantly, of pronounced slender and stout type (figs. 56–60). In one smear 120 stout and 60 slender were counted; in another, 78 stout, 34 slender; no dividing forms were seen.

Nov. 20th.—One Mansonia was dissected, but no trypanosomes were found, and unfortunately no smears were made.

Tæniorhynchus Experiments.

First batch, Nov. 19th.—Four mosquitoes (Tæniorhynchus sp.) fed on Monkey 478.

Nov. 20th.—Two dissected; in each active trypanosomes were noted. In the smears numerous typical slender and stout forms were found (figs. 61–65). Very few were dividing. In one smear were counted 78 stout, 41 slender forms (of which 4 were dividing); in another smear, 89 stout, 45 slender (1 dividing).

Nov. 21st.—Two dissected; a few rare active trypanosomes found, others motionless, apparently dead. In the smears made a fair number of trypanosomes were found, mostly of stouter form, often very vacuolated, and with *N* broken up (figs. 81–82); one slender form, apparently much macerated, was found. No dividing forms were seen.

Second batch, Nov. 20th.—A number of Tæniorhynchus fed on Monkey 478. Some were dissected after forty-eight hours (Nov. 22nd); in one a motile trypanosome was seen, but in the smear made from this none were found. The four remaining were dissected after seventy-two hours (Nov. 23rd); the blood had become reduced to a mass of dancing granules, amongst which blood-corpuscles were absent or very rare. In three of the mosquitoes no trypanosomes were found; in the fourth some active trypanosomes of stout type were found, but in the smear made none were found.

The experiments with Stomoxys and mosquitoes were highly incomplete, and it is to be regretted that more were not carried out; but so far as they go they show one interesting result, namely that the trypanosomes become differentiated into exactly the same two forms, stout and slender, as they do in Glossina. Further, the trypanosomes in the digestive tract one day after feeding appeared to have multiplied, as judged both by their number and by the frequent occurrence of dividing forms.

Examination of Lice from Sleeping Sickness Patients.

The Father Superior of the Sleeping Sickness Refuge, conducted by the Algerian White Fathers at Kisubi, kindly caused lice to be collected for me from patients in three degrees of the disease—early, medium, and late. I dissected ten lice of each batch and examined all their organs very carefully. The gut contained in all cases numerous cocci of fair size, in couples or clusters; the hinder part of the intestine frequently contained very large, active, rod-shaped bacilli. No trypanosomes were found in any case.

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APPENDIX.

Since the above was written a detailed memoir[2] on the cytology of trypanosomes has been published by Salvin-Moore and Breinl, in which the authors take a very different view of the nature of the nuclear apparatus from that taken by me. They regard the kinetonucleus as an "extra-nuclear centrosome" or blepharoplast, comparable with an intra-nuclear centrosome or karyosome contained within the principal nucleus. The extra-nuclear centrosome is described as "a granule or small group of granules which stain like the intra-nuclear centrosome," and the flagellum is stated to arise from one or more of these granules. In development the extra-nuclear centrosome is stated to arise from the intra-nuclear centrosome, which divides into two halves within the nucleus, after which one half is extruded from the nucleus to form the extra-nuclear centrosome.

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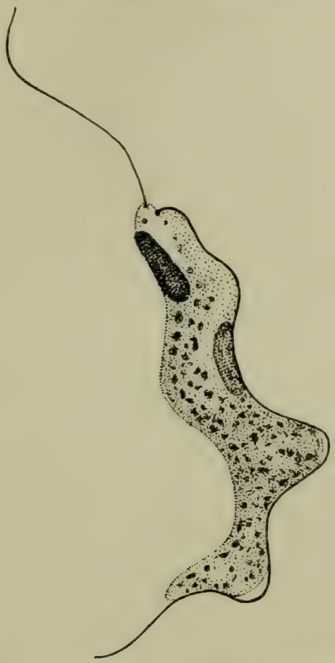
[1] This title is somewhat misleading; the author states (p. 83), "I have never seen any sign of an intra-corpuscular stage of the trypanosoma."

[2] Salvin-Moore, J. E., and Breinl, A., "The Cytology of the Trypanosomes," Part 1, 'Ann. Trop. Med. Parasitology,' vol. i, pp. 441–480, Pls. xxxviii–xlii.

I am unable to agree with Salvin-Moore and Breinl in regarding the kinetonucleus as of centrosomic nature. On the contrary, I agree with the recently expressed view of Keysselitz, to the effect that "Der Blepharoplast stammt vom Kern ab und besitzt Kernnatur" ('Arch. Protistenkunde,' x, p. 129); I should differ from Keysselitz in the use of the word blepharoplast, which, in my opinion, should be used for bodies of centrosomic and not of nuclear nature. I am of opinion, further, that Salvin-Moore and Breinl have confused two distinct structures under the name "extra-nuclear centrosome"; namely, the kinetonucleus, a chromatic body, and the true blepharoplast, a centrosomic or achromatic body. It



TEXT-FIG. B.—Trypanoplasma sp. from a pike (Esox lucius), Sutton Broad, Norfolk, to show the kinetonucleus, larger and more darkly stained than the trophonucleus, and the two blepharoplasts, from each of which a flagellum arises; the cytoplasm contains numerouse oarse staining granules. Osmic vapour, Giemsa, × 2000 linear.

is unfortunate from this point of view that these investigators have studied only pathogenic trypanosomes (T. gambiense, brucii, and equinum), in which the kinetonucleus happens to be extremely minute and does not contrast sharply with a blepharoplast; a peculiarity which may be characterised as a "morphological curiosity." If they had studied forms with a large kinetonucleus, like T. grayi, I do not think they would have fallen into this error. Still more decisive against the view of Salvin-Moore and Breinl, it seems to me, is the genus Trypanoplasma (text-fig. B), in which *n* is as large as or even larger than *N*, and in which two distinct blepharoplasts, from each of which a flagellum arises, can be made out plainly in good preparations; pace Salvin-Moore and Breinl, who would not concede, probably, that good preparations can be made anywhere but in Liverpool. Finally, I am unable to agree that a structure of the same nature and reactions as the kinetonucleus exists in the interior of the trophonucleus. Here, again, a form such as T. grayi is very instructive; the large kinetonucleus of this form stains very intensely and of a different tint from the granules composing the nucleus. If a structure similar to the kinetonucleus were contained in the nucleus it would be impossible to overlook it. After what I have said above it is not necessary to point out that for Trypanoplasma the notion that the trophonucleus contains a body similar to the kinetonucleus becomes an absurdity.

The difference, however, between the views of Salvin-Moore and Breinl and of myself is largely one of terminology, since the Liverpool investigators admit that the kinetonucleus arises from the nucleus. They describe it as originating by division of a karyosome, which they compare to the karyosome described by Schaudinn, in Coccidium schubergi; a karyosome of this kind is a chromatic body, even if it contain a centrosome. Further, Salvin-Moore and Breinl regard the two nuclei (*n* and *N*) of a trypanosome as comparable to two differentiated gamete-nuclei, a comparison which I consider far-fetched and misleading in the highest degree, but which at least shows that they regard both n and N as bodies of the nature of true nuclei. I hold that the terms centrosome and blepharoplast should not be applied to bodies which are recognised to be of the nature of nuclei; I differ, therefore, from the Liverpool investigators, as from Keysselitz, mainly in a matter of the use of words.

The memoir of Salvin-Moore and Breinl confirms me in the view I have expressed above; namely, that the body of a trypanosome contains two distinct nuclei, and that each of these two nuclei has a centrosomic corpuscle in connection with it; for that, in connection with the trophonucleus, I use the term centrosome simply, since its function is mainly related to division of the nucleus; for that in connection with the kinetonucleus I use the term blepharoplast, since the flagellum takes origin from it. I can imagine that this type of structure may be susceptible of variations and additions; the centrosome might be imbedded in a chromatic mass or true karyosome; the blepharoplast might be lodged in the centre of the kinetonucleus; in either case the essential nature of blepharoplast and centrosome would not be affected.

With reference to my diagram given above (Text-fig. A, p. 174), I should explain that I have purposely given a negative picture, so to speak, of the nucleus and centrosome; that is to say, I have represented the centrosome as a distinct black granule in the midst of colourless chromatic granules making up the trophonucleus; had I represented the centrosome as it really is, namely, as a colourless granule in the midst of deeply staining chromatin-granules, it would have been as difficult to see in my drawing as it usually is in the actual preparations of trypanosomes.

Salvin-Moore and Breinl deny any differentiation of trypanosomes in the blood; they state that the three types, slender, intermediate, and stout, distinguished by me, are "arbitrarily chosen examples in a continuous series of dimensions." To this I reply, first, that it has never been disputed that the different types are connected by transitions, since both the slender and stout forms are differentiations, more or less strongly marked, of the common intermediate type; secondly, that the difference between the extreme forms is not one of dimensions alone, but of points of structure and morphology which Salvin-Moore and Breinl have overlooked. I am content to let my figures speak for themselves.

Since my work relates almost exclusively to the development of Trypanosoma gambiense in the tsetse-fly, I have no comment to offer on the cycle in the blood, with formation of latent forms, described by Salvin-Moore and Breinl; a discovery of the highest importance, if true, but which does not, in my opinion, disprove the existence of a developmental cycle in an invertebrate host. It is possible, however, that the pathogenic trypanosomes as a group may owe their peculiar properties to having become adapted exclusively to vertebrate hosts; but in that case the problem of transmission and infection becomes difficult to understand. Salvin-Moore and Breinl state (p. 446) that the blood during negative phases, "even if it be properly filtered, is still capable of infecting." This is a very important statement, and I look forward with much interest to the publication by the authors of the evidence on which it is based. At present they have given us none.

LISTER INSTITUTE, January 15th, 1908.

DESCRIPTION OF PLATES 8—13.

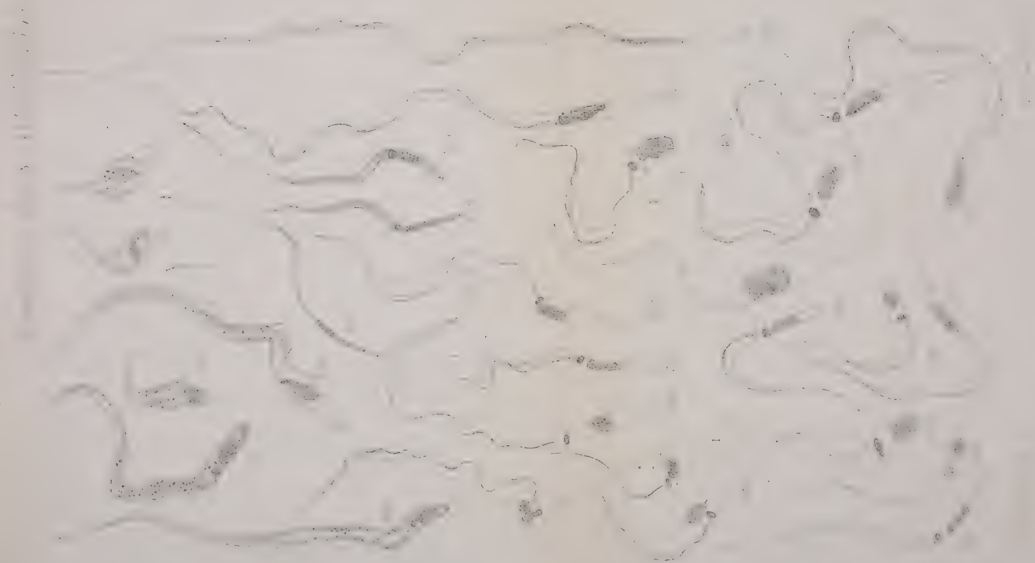
Illustrating Prof. E. A. Minchin's paper on "Investigations on the Development of Trypanosomes in Tsetse-Flies and other Diptera."

All the figures are drawn with the camera lucida to a magnification of 2000 diameters, except Figs. 102–110, Figs. 122–125, and Figs. 127–130 on Pl. 10, which are freehand sketches.

PLATE 8.

Trypanosoma gambiense.

FIGS. 1–5.—From the blood of Monkey 478, on October 1st, 1905 (pp. 176 and 230).



Figs. 6–10.—From the blood of Monkey 478, on October 2nd, 1905 (pp. 176 and 231).

Figs. 11–13.—From the blood of Monkey 478, on October 19th, 1905 (pp. 176 and 237).

Figs. 14–17.—From the blood of Monkey 478, on November 3rd, 1905 (p. 239).

Figs. 18–21.—From the blood of "fresh-fly monkey" (p. 175).

Figs. 22–25.—From the blood of Monkey 420, on July 16th, 1905 (p. 226).

Figs. 26–29.—From the blood of male Chimpanzee, eighteen days after inoculation with cerebro-spinal fluid.

Figs. 30–35.—From human cerebro-spinal fluid.

Figs. 36–39.—From the stomach of Glossina palpalis one hour after feeding on an infected monkey, on July 31st, 1905 (p. 228).

Figs. 40–44.—From the stomach of G. palpalis five hours after infection on July 31st, 1905 (p. 228).

Figs. 45–47.—From G. palpalis twenty-four hours after infection, on October 16th, 1905 (p. 234); Fig. 46 from the red blood; Figs. 45 and 47 from the black blood.

Figs. 48–50.—From G. palpalis twenty-four hours after infection, on November 4th, 1905 (p. 240); red blood from second fly.

Figs. 51–55.—From Stomoxys sp. twenty-four hours after infection, on November 18th, 1905 (p. 247); Figs. 51 and 52 from one fly; Figs. 53–55 from another.

Figs. 56–60.—From Mansonia sp. twenty-four hours after infection, on November 20th, 1905 (p. 247).

Figs. 61–65.—From Tæniorhynchus sp. twenty-four hours after infection, on November 20th, 1905 (p. 247).

PLATE 9.

Trypanosoma gambiense.

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Figs. 69–72.—From G. palpalis forty-eight hours after infection, on October 17th, 1905, third fly (p. 235); Fig. 60, red blood; Figs. 70–72, black blood.

Figs. 73–77.—From G. palpalis forty-eight hours after infection, on October 17th, 1905, first fly (p. 234); Figs. 73–75, black blood; Figs. 76, 77, red blood.

Figs. 78–80.—From G. palpalis forty-eight hours after infection, on October 21st, 1905, first, second, and fourth flies respectively (p. 237).

Figs. 81, 82.—From Tæniorhynchus sp. forty-eight hours after infection, on November 21st, 1905 (p. 248).

Figs. 82 *a*–85.—From Glossina palpalis seventy-two hours after infection, on October 18th, 1905, third fly (p. 236); Fig. 82 *a*, red blood; Figs. 83–85, black blood.

Figs. 86–88.—From G. palpalis seventy hours after infection, on October 18th, 1905, 2nd fly (p. 236).

Figs. 89–92.—From G. palpalis seventy-one hours after infection, on October 22nd, 1905, second fly (p. 238).

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Trypanosoma gambiense; other parasites of Glossina palpalis.

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Figs. 144–150.—From November 10th, 1905, second fly, proctodæum (p. 241).

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Figs. 156–164.—From November 13th, 1905, third fly, black blood (p. 243).

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- Figs. 187, 188.—Herpetomonas-like forms, free, not begun to form cysts.
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- Figs. 194–198.—Forms in which the retractation of the flagellum and the formation of the cyst is more advanced. Figs. 194 and 195 show the condition with a flagellum vacuole.
- Figs. 199–202.—Ripe cysts. Fig. 202 is from a thin smear and is damaged; the others are from a thick smear. In Figs. 200 and 201, *n* cannot be identified with certainty.
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PLATE 13.

Trypanosoma grayi from Glossina palpalis.

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Figs. 234–244.—From November 2nd, 1905 (p. 239).

UPON THE BACTERIOLOGY OF THE SUMMER DIARRHOEA OF INFANTS.

[Second Communication]

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Summary of Last Year's Report.

In my previous report, which was published in the British Medical Journal of April 21st, 1906, a detailed account was given of my investigations into the bacteriology of the summer diarrhoea of infants in London during the course of the summer of 1905.

The non-lactose fermenting bacteria isolated from the stools or intestines of 58 cases of summer diarrhoea were compared with those isolated from the stools of 20 normal infants under 2 years of age. By this comparison it was possible to show that a certain bacillus pathogenic to animals, which has been designated by me as "No. 1 bacillus," was isolated in 28 out of the 58 cases of summer diarrhoea, whereas that bacillus was found in the stools of only 1 of the 20 normal cases, and even then its identity was doubtful, as in that case it was found to be non-pathogenic for experimental animals. The pathogenicity of Bacillus No. 1, which was isolated from cases of summer diarrhoea, was then tested by feeding experiments. A series of young rats and young rabbits were fed on various strains of the bacillus, half an agar tube being used in the case of the former, and one tube in the case of the latter, with the result that all the animals were attacked with diarrhoea and died within a few days, the bacillus being recovered from their spleens after death.

I made inquiries from Dr. Houston and also from Dr. MacConkey, both of whom had made extensive bacteriological examinations of water, sewage, human faeces and milk, as to whether either of them had ever isolated this bacillus from any of the above sources. They both very kindly looked up their notes, and were able to assure me that they had never isolated any such bacillus, and so far as I can ascertain the bacillus has not hitherto been described.

The characteristics of this bacillus are very distinctive. It is a motile rod about the size of the *Bacillus enteritidis* of Gaertner, it is non-sporebearing, does not liquefy gelatin, and gives a negative reaction on broths containing mannite, dulcite, lactose and cane sugar, but produces acid and a small amount of gas on glucose broth. The fact of its producing acid and gas on glucose broth, and not on mannite broth, brings this bacillus into a comparatively small group, the only important pathogenic member of which is the hog cholera bacillus of McFadyean from which, however, it is easily differentiated by other tests.

These results aroused the suspicion that this bacillus might be an important factor in the causation of the summer diarrhoea of infants, and this suspicion was strengthened by the fact that it was isolated in pure culture from the stools of one of the nurses who had contracted diarrhoea in the ward set apart for this disease in the Hospital for Sick Children, Great Ormond Street.

In view of the importance of the results obtained from one year's investigations, it seemed advisable to continue the work on the same lines during another summer epidemic. I therefore wrote to the authorities at the Hospital for Sick Children, Great Ormond Street, who very kindly promised to supply me with specimens for another year. Dr. Batten also undertook to supply the stools and *post-mortem* specimens from the ward set apart for diarrhoea at that hospital, independently of whether he diagnosed them as suffering from summer diarrhoea or not, at the same time withholding his diagnosis until after the bacteriological examination.

THIS YEAR'S RESEARCH.

During last summer (1906) 54 cases from the special ward set apart for diarrhoea at the Hospital for Sick Children, Great Ormond Street, were examined bacteriologically in the same manner as during the previous year, except that on this occasion the spleens and mesenteric glands were examined as well as the stools and small and large intestines.

METHODS ADOPTED FOR THE ISOLATION OF THE BACTERIA.

A small portion of material, either from the faeces or scrapings from the mucous surface of the small or large intestine, was transferred to a tube of sterile peptone beef broth and an emulsion made; from this the bile-salt-neutral-red-lactose-agar plates of MacConkey were inoculated, and incubated for twenty-four hours at 37° C. In the examination of the spleens and mesenteric glands, the outside of these organs was previously sterilized in a Bunsen flame, and a small portion taken from the inside and rubbed direct on to the lactose agar plates, which were then incubated. On the following day all the colourless colonies—that is, the non-lactose fermenters—were picked off, and put into tubes of lactose broth. These tubes were then incubated for five days at 37° C., at the end of which time all those that had not produced acid and gas were retained and the rest discarded. The former were then used to inoculate gelatine tubes, and set aside for future investigation. In the numerous instances where no colourless colonies were to be found on the agar plates, the same material, which had been kept frozen in the cold room, was used for reinoculating fresh agar plates.

It was thought advisable to continue the use of the lactose agar plates this year, in order to isolate as many of the non-lactose fermenters as possible, so as to compare the relative frequency of each species. If, however, Bacillus No. 1 had been looked for exclusively, mannite agar plates would have been more serviceable, since that bacillus does not ferment mannite. But if this method had been adopted, the frequent occurrence of some other organism might have been overlooked, since the non-mannite fermenting group of bacilli is relatively smaller than the non-lactose fermenting one.

The next step was to examine all the cultures that had not liquefied gelatine at the end of six weeks, and to cultivate them on the various media used for differentiation —namely, broth containing glucose, mannite, dulcite, lactose, cane sugar, and litmus milk—to examine their morphology, motility, etc., and their capability of producing indol in peptone beef broth when incubated for five days.

In Table I is shown the results of the examination of the morphology and cultural characteristics of sixty-seven organisms of the lactose non-fermenting class isolated from the spleens, mesenteric glands, intestines, or faeces of infants suffering from diarrhoea.

Table II gives the characteristics of some of the known pathogenic intestinal bacteria.

Bacillus No. 6.—It will be seen on comparing the two tables that this bacillus, which was isolated from four patients after death, resembles the *Bacillus enteritidis* of Gaertner, and it was subsequently proved by its agglutination reactions to belong to this group.

Bacilli Nos. 3 and 4—There is a partial resemblance between these bacilli and the Philippine dysentery bacillus of Flexner. No. 3, however, differs in its reaction on sorbite, on which it produces acid, whereas Flexner's bacillus does not do so, and No. 4 does not produce indol, whereas Flexner's bacillus gives a well-marked indol reaction. Bacilli Nos. 3 and 4 also both fail to agglutinate with dysentery serum (Flexner).

Bacillus No. 5 resembles the *Bacillus dysenteriae* of Shiga in its cultural reactions, but differs in its distinct motility and its absence of agglutination with antidysentery serum (Shiga).

Bacillus No. 1, as was pointed out in my last year's report, resembles no other known pathogenic organism, except the bacillus of hog cholera of McFadyean, from which it differs, however, in its alkaline reaction on litmus milk, its greater production of indol, and its failure to produce acid and gas on arabinose, maltose, and dextrin.

TABLE I.

Nomenclature.	Morphology.	Glucose.	Mannite.	Dulcite.	Lactose	Cane Sugar.	Litmus Milk.			Indol.	Sorbite.	Gelatine	No. of Cases in which Found.
							1 Day.	3 Days.	15 Days.				
1	M. B.	A. G	—	—	—	—	0	0	Alks.	+	—	—	19
3	N. M. B.	A.	A.	—	—	—	0	0	0	+	A.	—	3
4	N. M. B.	A.	A.	—	—	—	0	0	0	—	—	—	1
4A	N. M. B.	A.	A.	—	—	—	A.	A.	A. C.	—	—	—	5
4B	M. B.	A.	A.	—	—	—	A.	0	Alk.	+	—	—	1
4C	M. B.	A.	A.	—	—	—	A.	0	Alk.	—	—	—	1
5	M. B.	A.	—	—	—	—	0	0	Alks.	+	—	—	4
6	M. B.	A. G.	A. G.	A. G.	—	—	A.	0	Alk.	—		—	4
7	M. B.	A. G.	A. G.	A. G.	—	—	A.	Alk.	Alk.	+		—	4
7A	N. M. B.	A. G.	A. G.	A. G.	—	—	A.	0	A. C.	+		—	1
10	N. M. B.	A. G.	A. G.	A. G.	—	A. G.	A.	A.	A. C.	+		—	1
11	M. B.	A. G.	A. G.	A. G.	—	A. G.	0	0	Alk.	+		—	2
11A	M. B.	A. G.	A. G.	A. G.	—	A. G.	A.	A.	Alk.	—		—	3
11B	M. B.	A. G.	A G.	A. G.	—	A. G.	0	A.	A. C.	+		—	1
13	M. B.	A. G.	A. G	—	—	A. G.	A.	A.	A. C.	+		—	3
13A	N. M. B.	A. G.	A. G.	—	—	A. G.	A.	A.	A. C.	+		—	2
14	M. B.	A. G.	A. G.	—	—	—	A.	A.	Alks.	+		—	1
14A	N. M. B.	A G.	A. G.	—	—	—	A.	Alk.	Alk.	+		—	3
14B	M. B	A. G.	A. G.	—	—	—	A.	A.	A. C.	+		—	3
14C	N. M. B.	A G.	A. G.	—	—	—	A.	0	A. C.	+		—	2

TABLE II.

Bacterium.	Morph.	Glucose.	Mannite.	Dulcite.	Lac-tose.	Cane Sugar.	Litmus Milk.			Litmus Whey.		Indol.	Sor-bite.
							1 Day.	3 Days.	15 Days.	1 Day.	7 Days.		
B. dysentery, Flexner, Philippines	B. Non-motile	A.	A.	—	—	—	A.	Alks.	Alks.	A.	Alks.	+	—
B. dysentery, Shiga ...	,,	A.	—	—	—	—	A.	Alks.	Alks.	A.	Alks.	—	
B. typhoid	B. Motile	A.	A.	—	—	—	A.	A.	A.	A.	A.	—	A.
B. enteritidis, Gaertner...	,,	A. G.	A. G.	A. G.	—	—	A.	Alk.	Alk.	A.	Alk.	—	
B. paratyphoid B, Schottmüller	,,	A. G.	A. G.	A. G.	—	—	A.	Alk.	Alk.	A.	Alk.	—	
B. paratyphoid A, Schottmüller	,,	A. G.	A. G.	A. G.	—	—	A.	A.	A.	A.	A.	—	
B. hog cholera, McFadyean	,,	A. G.	—	—	—	—	A.	A.	A.	A.	A.	+ Slight	

A. = Acid. G. = Gas. C. = Clot. S. = Slight. — = No reaction. M. B. = Motile bacillus. N. M. B. = Non-motile bacillus.

As regards the cultural reactions of No. 1, a considerable amount of variation was found in the degree of alkalinity produced in various batches of litmus milk. The amount of gas produced in glucose broth also varied considerably certain strains of this bacillus producing so little gas that the Durham's tube failed to catch any, and it was only by making a glucose agar shake culture that the production of gas could be determined.

The use of glucose agar shake cultures seems to be important when looking for this organism, in order to differentiate it from Bacillus No 5, which culturally closely resembles it, except that it produces acid, but no gas on glucose. The gas-producing power of Bacillus No. 1 on glucose media seems to be decreased by its being kept for some time on artificial media. It has been observed that cultures which shortly after their isolation gave a measureable amount of gas in the Durham's tube failed to do so after eighteen months' growth on artificial media. When, however, agar-shake cultures were made from them, their gas production became quite evident.

MATERIAL FROM WHICH BACILLI NOS. 1 AND 6 WERE ISOLATED.

Bacillus No. 1 was isolated from the stools of 16 cases, from the scrapings of the large intestines of one case, from the scrapings of the large and small intestines of one case, and from the mesenteric glands and large and small intestines of another. It was never found in the spleen of any case examined, which was disappointing in so much as it had been invariably found present in the spleens of young rats and young rabbits fed on it experimentally. It will be seen later, however, that invasion of the mesenteric glands and spleen did not occur in feeding experiments on monkeys.

Bacillus No. 6, or the Gaertner type of bacillus, was isolated from the spleens and mesenteric glands in 3 cases, and from the spleen only in the fourth case.

RELATIVE FREQUENCY OF THE BACILLI ISOLATED: PREPONDERANCE OF BACILLUS NO. 1.

Material from 54 cases was examined, and of these 27 had been diagnosed clinically as being due to acute infective diarrhoea, 7 to catarrhal diarrhoea, 2 to Gaertner infections, and the remaining 18 to various ailments complicated with diarrhoea.

Bacillus No. 1 was isolated from 12 out of the 27 acute infective diarrhoea cases, and from 3 of the 7 catarrhal diarrhoea cases; in other words, this bacillus was isolated in 15 out of 34 cases of summer diarrhoea, giving it a large preponderance over any other bacillus isolated. The bacillus which held the second place in frequency of occurrence was No. 4a, which was found in only 5 cases out of the 54 examined.

Bacillus No. 1 was also found in 4 out of the remaining 18 cases, the 4 in which it was found having been diagnosed clinically as being due respectively to marasmus, tuberculosis and diarrhoea, cleft palate and diarrhoea, and bronchopneumonia and diarrhoea. Considering, however, that these 4 cases were complicated with diarrhoea, and were taken from the ward set apart for that condition, it is not surprising that this bacillus should have been isolated from their stools.

TABLE III.—*Agglutination of Various Strains of Bacillus No. 1, with Serum Agglutinating Strain 33 M in a Dilution of 1 in 2,000.*

Strain of Bacillus	Dilution of 1 in 20.	Dilution of 1 in 200.	Dilution of 1 in 2,000.
33 M	3	3	3
7	3	3	0
12	3	3	0
15	3	3	2
16	3	0	0
19	0	0	0
22	3	2	0
26	0	0	0
28	0	0	0
30	0	0	0
35	3	3	2
36	3	0	0
41	0	0	0
44	0	0	0
46	3	3	0
47	3	3	0
51	3	3	2
52	3	3	2

3 = Complete reaction ; 2 = distinct reaction ; 1 = slight reaction.

AGGLUTINATION REACTION EXPERIMENTS.

The agglutination of certain of the bacilli was tested with serums obtained from animals immunized with known pathogenic micro-organisms.

Bacilli Nos. 3 and 4 were tested with the Lister Institute dysentery serum, which gives a reaction with dysentery bacilli in a dilution of 1 in 100,000; but no reaction was obtained with either No. 3 or No. 4 in a dilution of 1 in 1,000, which, together with their slight difference in cultural characteristics from Flexner's dysentery bacilli, excluded the possibility of their belonging to that group.

The four strains of Bacilli No. 6 were next tested against the serums of three rabbits immunized against the *Bacillus enteritidis* of Gaertner, the *Bacillus enteritidis* of Aertrycke, and the *Bacillus paratyphoid* B. of Schottmüller respectively. These serums agglutinated their own bacilli in a dilution of 1 in 2,000. Two of the strains of No. 6 gave a distinct reaction with Gaertner serum, and two with the Aertrycke serum, in a dilution of 1 in 2,000, but all failed to give any reaction with the paratyphoid B. serum.

The inter agglutination of the various strains of Bacillus No. 1 was next investigated. A rabbit was immunized by intravenous injection with the bacillus isolated from Patient No. 33, and a serum was obtained which agglutinated this bacillus in a dilution of 1 in 2,000.

It will be seen from Table III that of the 17 strains of Bacillus No. 1 which were investigated, 4 were agglutinated in a dilution of 1 in 2,000, 5 in a dilution of 1 in 200, 2 in a dilution of 1 in 20, and 6 failed to react at all. Very similar results to these were obtained last year from the various strains of this bacillus isolated during the previous summer.

TABLE IV.

No. of Case from which Bacillus was Isolated.	Experimental Animal.	Dose.	Diarrhoea.	Died No. of Days after Experiment.	Bacillus Recovered from Spleen.
7	young rat	½ agar tube	?	8	0
12	,,	,,	?	9	0
15	,,	,,	+	19	+
16	,,	,,	?	12	0
19	,,	,,	+	3	0
20	,,	,,	+	11	0
22	,,	,,	+	2	+
26	,,	,,	+	11	+
28	,,	,,	+	10	+
30	,,	,,	+	3	+
33	,,	1 agar tube	+	1	+
35	,,	,,	+	1	+
36	,,	,,	+	1	0
41	,,	,,	?	lived	—
44	,,	,,	?	6	0
46	,,	,,	+	1	0
47	,,	,,	+	1	+
61	,,	,,	+	2	+
52	,,	,,	?	lived	—

THE PATHOGENICITY OF THE BACILLI ISOLATED FROM THE PATIENTS.

One of the two bacilli which were so readily agglutinated by the serum made by the injection of Aertrycke bacilli, and classed under No. 6, was injected subcutaneously into a rabbit in the dose of ¼ c.cm. of a 24-hours old broth culture, for the purpose of immunization, with the result that the rabbit died on the sixth day, and the organism was recovered from the heart blood, thus proving that this organism is a virulent race of the Aertrycke bacillus.

In order to test the virulence of the various cultures of No. 1 bacillus, each of these was used for feeding experiments on young rats. Half an agar culture of each of the nine different strains of No. 1 was used for feeding nine young rats, one rat being used for each strain, and an equal number of control rats being kept. In the next experiment young rats were fed on the remaining ten strains of No. 1, a whole agar culture in this case being used for each rat, an equal number of control rats being kept. Table IV shows the result of these experiments.

It will be observed that all the rats died with the exception of two, in which case the experiment was repeated three times with fresh rats with the same result, showing that these two strains of No. 1 were of a lower virulence for rats than the rest.

Diarrhoea was observed in 12 out of the 19 rats, but its occurrence in the others might easily have escaped detection. The bacillus was recovered from the rats' spleens after death in 9 cases out of the 19; it was not looked for in any of the other organs.

TABLE V.

No. of Case from which Bacillus was Isolated.	Experimental Animal.	Dose.	Diarrhoea No. of Days Before Death.	Died No of Days After Experiment.	Stools or Organs from which Bacillus was Recovered.
35 R	Monkey	1 agar tube	1 day	3	Bile.
35	,,	,,	2 days	12	Faeces and small intestine.
30	,,	,,	6 days	8	Faeces 3rd and 7th day. large and small intestines.
52	,,	,,	3 days	15	Faeces 13th day.

It was then thought that monkeys, being more closely allied to human beings, might be more suitable than rats as experimental animals. Four small full-grown monkeys were fed on one agar tube of Bacillus No. 1. A different strain was employed for each monkey. The results of these experiments are shown in Table V.

It will be observed that the onset of the diarrhoea varied from two to twelve days after the feeding. The bacillus was recovered from all four of the animals after the onset of the diarrhoea, from three after death, and from the faeces of three after the onset of the diarrhoea. The bacillus was not found in the spleens of these animals, a condition analogous to that found in infants suffering from summer diarrhoea.

It will be seen that Strain 52, which proved non-virulent for rats, produced diarrhoea and death in a monkey, the only difference from the other strains being that the incubation period before the onset of the diarrhoea was longer.

The condition produced in monkeys by these feeding experiments closely resembled infective diarrhoea in infants, the diarrhoea being acute and progressive in severity, accompanied by rapid emaciation followed by death. No vomiting was observed in any of the monkeys. Control monkeys kept under identical conditions remained quite healthy all the time.

The virulence of Bacillus No. 1 was also inadvertently tested on a goat, which for the purpose of immunization had been injected intravenously with an agar culture of a strain of that bacillus, which had been isolated from the mesenteric glands of a child that had died of acute infective diarrhoea. The goat died in about eighteen hours, and the bacillus was recovered from the heart blood, spleen, and liver. Considering that a rabbit not one-twentieth part of the weight of this goat had been successfully immunized with half the dose of the same culture of Bacillus No. 1 intravenously injected, this experiment points to the much greater susceptibility of the goat to this organism.

CONCLUSIONS.

In the course of the present investigation into the bacteriology of summer diarrhoea, there has been isolated a bacillus, designated Bacillus No. 1, which, so far as I have been able to ascertain, has not hitherto been described, and which appears to me to be entitled, in the absence of further knowledge, to be regarded as a factor, perhaps the most important factor, in the causation of the disease. The reasons on which this conclusion is based are as follows:

It has never been isolated in any other morbid condition, nor has it been observed in water, milk, sewage, or human faeces.

In all the cases of the disease examined during two consecutive summers, it was found to preponderate in frequency over all other non-lactose fermenting bacilli—for example, out of 58 cases examined during the summer and autumn of 1905 it was isolated from 28, and out of 34 cases in 1906 it was isolated from 15 cases.

It is pathogenic for animals, producing diarrhoea and death in young rabbits, rats, and monkeys, when these animals are experimentally fed on cultures.

It differs from the bacillus of hog cholera of McFadyean, to which it appears to be most closely allied, in its reaction on litmus milk, in the production of a larger amount of indol, and in its failure to produce acid and gas on arabinose, maltose, and dextrin.

It is probable that the difficulties of clinical diagnosis in such a condition lead to the classification under one name of cases due to different micro-organisms. An analogy to this is seen in the case of enteric fever, under which all cases now designated as paratyphoid were till recently included.

It is improbable, from the bacteriological standpoint, that a sharp line of demarcation should be found to exist between acute infective diarrhoea and catarrhal diarrhoea in infants, and this is borne out by the results obtained in the present investigation.

In one case of acute infective diarrhoea the *Bacillus enteritidis* of Gaertner was isolated from the spleen and mesenteric glands, and it seems probable that other allied micro-organisms which produce a condition with even less marked clinical features (than does that organism) may not infrequently be involved in the production of what is designated clinically "infective diarrhoea of infants."

It is remarkable that again this summer no bacilli of the true dysentery Flexner type were found, whilst Duval and numerous workers at the Rockefeller Institute found that organism to be the causal agent of the disease in America.

It is true that bacilli somewhat resembling *Bacillus dysenteriae* (Flexner) were isolated from four cases, but they were not culturally identical with any of the various types of this bacillus, nor were they agglutinated by anti-dysentery (Flexner) serum.

This emphasizes what was said in my last year's communication, that the type of summer diarrhoea of infants in America appears to be clinically and bacteriologically different from that which occurs in this country.

My thanks are due to Dr. Batten, who took the greatest interest in this research, and for the second summer supplied me with the material from the Hospital for Sick Children, Great Ormond Street; also to Dr. Dean and Dr. Boycott, of the Lister Institute, for their most valuable assistance and advice.

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Further Observations on the Effects produced on Rats by the Trypanosomata of Gambia Fever and of Sleeping Sickness.

By H. G. PLIMMER, F.L.S.

(Communicated by Dr. C. J. Martin, F.R.S. Received November 21,—Read December 13, 1906.)

[PLATE 1.]

In January, 1905, I published* a note of some experiments with the above-named strains of trypanosomata, and I have continued the experiments up to the present time, and have embodied the total results in this paper.

1. *Gambia Fever.*

The number of rats inoculated with this strain of trypanosomata was altogether 129 in 90 successive inoculations. In all of these rats, with the exception of the five mentioned below, trypanosomata were present in the blood from two to three weeks after inoculation (average time 18 days) until death; generally in good numbers, and increasing to large numbers for some days before death. The spleen was, in these cases, very much enlarged, and there was considerable blood destruction, with large numbers of polychromatophile corpuscles, these being the most obvious gross signs.

In the first 14 of these rats, as noted in the paper referred to above, no paralytic symptoms were present, but out of the total number inoculated, five became paraplegic, and the course of the disease in these animals differed from that in the remaining 124.

In the series, the numbers of the paraplegic rats were 35, 42, 68, 101, 102, and there were no perceptible differences in their conditions and those of the other rats used.

The first rat which became paralysed (No. 35, of the 25th inoculation) was inoculated on March 17, 1905, and on the 31st of the same month both hind legs became paralysed. No trypanosomata were found in the blood until April 18, and from that time till death, which took place on April 28, only a few were to be found in ordinary blood films. The second rat (No. 42, of the 28th inoculation) was inoculated on April 13, 1905, from the blood of another rat which had been 20 hours in the stomach of a leech, in which some of the trypanosomata will live for at least 36 hours. The rat became paraplegic on June 15, at which time no trypanosomata could be found in ordinary blood films. The rat was killed on June 16, and a few trypanosomata were found both in the centrifugalised blood and in smears made from the spinal cord. The third rat (No. 68, of the 42nd inoculation) was inoculated on September 26, 1905, and became paraplegic on December 13. No trypanosomata could be found in ordinary blood films in 17 examinations made during the disease, nor at the time of death. The animal died on January 3, 1906, when a few trypanosomata were found in the centrifugalised blood, and in smears from the spinal cord. The fourth and fifth rats (Nos. 101 and 102, of the 70th inoculation) were inoculated on May 26, 1906, and on July 17 No. 101 became paralysed in the right hind leg, and on July 18 No. 102 also became paralysed in the right hind leg. Both of these rats had a good number of trypanosomata in their blood of the ordinary long, very motile kind. On July 19 No. 101 became completely paraplegic, and died on the 22nd. No. 102 died on July 23, without the other leg becoming paralysed.

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* 'Roy. Soc. Proc.,' vol. 74.

The following table gives a comparative outline of these five experiments:—

Rat.	Duration of disease.	Appearance of paralytic symptoms.	Trypanosomata in blood during life.
35	41 days	14th day	Very scanty.
42	64 days, rat killed...	63rd day	Not found; few found *post mortem* in centrifugalised blood.
68	102 days	78th day	Ditto.
101	57 days	52nd day	Good number.
102	58 days	53rd day	Ditto.

It will be noted that, in Rats Nos. 101 and 102, trypanosomata were present in good numbers in the blood, but in those cases (Nos. 42 and 68) in which the paralysis was most marked, and which, to all appearances, were exactly like rats paralysed after inoculation with the Sleeping Sickness strain of trypanosomata, they were absent, or at any rate so few in number that they could only be found in centrifugalised blood after death. From all of these rats inoculations were made, with the constant result that the disease in the inoculated animals reverted to the generalised form mentioned above as characteristic of this strain of Gambia Fever.

This strain of trypanosomata was also inoculated into a monkey, which lived 33 days after inoculation, this being three days longer than the average time of the 14 monkeys I have inoculated with the Sleeping Sickness strain. Trypanosomata appeared in the blood 10 days after inoculation, and were of the type of the Sleeping Sickness strain. Rats inoculated from this monkey did not become paralysed; trypanosomata of the Gambia Fever type were plentiful in their blood, and the disease ran the course of that produced by this strain.

2. *Sleeping Sickness.*

In all, 82 rats have been inoculated with this strain of trypanosomata, in 59 direct series of inoculations, and, of these, 22 have been paralysed. In these cases trypanosomata could be found only at intervals and rarely, and sometimes not at all, in ordinary blood films, and in some cases none could be found in the centrifugalised blood *post mortem*; but in cases where prolonged search was made a few were always found. The paralysis occurred most generally in those rats which were inoculated direct from monkeys, and it occurred less and less frequently as the passages got more remote, firstly from the monkeys and secondly from the original monkey. I have succeeded in producing paralysis once as far as the fourth passage from the monkey; generally after the second, in five instances after the first, passage the trypanosomata have become generalised in the blood, and the symptoms and course of the disease have been those of the Gambia Fever strain.

The following table gives an outline of the instance mentioned above, in which paralysis occurred in four successive passages :—

Rats.	Inoculated from	Duration of disease.		Appearance of paralysis after inoculation.		Trypanosomata in blood.	Spleen.
		mths.	days.	mths.	days.		
7*a*	Monkey which was inoculated from the monkey brought home by Col. Bruce	6	7	5	15	None seen	Not enlarged.
7*b*		3	14	2	2	None seen	Not enlarged.
8	Rat 7*a*	6	0	5	2	None seen	Not enlarged.
10*a*	Rat 8	3	23	3	1	Few	Enlarged.
10*b*		4	6	3	0	Few	Enlarged.
13*a*	Rat 10*a*	3	3	Not paralysed		Few in every field	Large.
13*b*		2	0	1	20	Few	Large.
16	Rat 13*b*	2	15	Not paralysed		Plentiful	Very large.

The rats Nos. 7*a* and 7*b* at the head of the above series were inoculated from a monkey which had been inoculated from the monkey Colonel Bruce, F.R.S., brought home from Uganda, this monkey having been inoculated on the spot with cerebro-spinal fluid from a case of advanced Sleeping Sickness.

In rats inoculated from the 10th monkey done in direct succession from Colonel Bruce's monkey, the paralysis occurred only in those inoculated directly from the monkey, the next passage producing trypanosomata and symptoms of the Gambia Fever type, as set forth in the following table :—

Rat.	Inoculated from	Duration of disease.	Appearance of paralysis after inoculation.	Trypanosomata in blood.	Spleen.
66*a*	Tenth monkey in direct succession from Col. Bruce's monkey	Killed at 3 mths. 25 days	2 mths. 22 days	None seen	Not enlarged.
66*b*		4 mths. 2 days	3 mths. 11 days	None seen	Not enlarged.
66*c*		4 mths. 6 days	3 mths. 22 days	None seen	Not enlarged.
68	Rat 66*a*	2 mths. 26 days	None	Few from 15th day after inoculation, increasing till death	Large.
69	Rat 66*b*	2 mths. 1 day	None	Plentiful	Very large.
70	Rat 66*c*	Killed at 1 mth. 20 days	None	Fair number	Large.
71	Rat 69	2 mths. 18 days	None	Plentiful	Very large.

3. *Paralysis after Inoculation with Trypanosomata.*

With regard to the question of paralysis occurring in the rat after inoculation with the above-mentioned particular strains of trypanosomata, it does not appear to have come within the experience of all those who have worked with these particular organisms. For instance, neither Dr. Thomas, of the Liverpool School of Tropical Medicine, nor Dr. Laveran, of Paris, appear to have encountered it in their work with these trypanosomata.

But Lieutenants Tulloch and Gray, in Uganda, have made a number of experiments to test the statement made in my Preliminary Note, and have recorded the same results in a paper published in No. VIII of the Sleeping Sickness Reports of the Royal Society.

In four rats inoculated with the blood of a monkey which had been previously inoculated with cerebro-spinal fluid from Sleeping Sickness patients, paralysis of the hind limbs occurred 10 or more days before death; and in three out of the four rats no trypanosomata could be found in the peripheral blood at the time of death. They also found that rats did not show any signs of infection after inoculation with the blood of Sleeping Sickness patients, which has also been my experience. I inoculated two rats with blood from a case of Sleeping Sickness, in University College Hospital, a year before death, and two more shortly before death, on both of which occasions trypanosomata were present in the patients' blood; the results in both series were negative.

The site of the inoculation apparently determines the locality of the paralysis. In all my experiments, with the exception of four, inoculation was made at the inner side of the hind leg, and the paralysis occurred in the lower limbs. Only in one rat, which lived nearly 11 months, did any weakness of the forelegs show itself; and this was due to extensive degeneration ascending the spinal cord from the lumbar region. The lesions in the spinal cord were also confined more or less to the lumbar region, depending for their extent upon the duration of the disease. In two cases I inoculated the animals on the inner side of the foreleg, and both these animals became paralysed in the front legs, and the degenerative lesions were most marked in the corresponding parts of the spinal cord.

I inoculated two rats in the peritoneum, but in neither did any paralysis occur.

4. *The morphological characters of these two strains of Trypanosomata.*

It is impossible at present to insist that any differentiation based solely upon microscopical observations should be sufficient in the case of organisms so much alike as the trypanosomata. Our methods and observations are neither uniform nor good enough to enable us to make, at the present time by the microscopical method alone, a sufficient differentiation. Until all observers use the finer zoological methods of fixation, etc.,* instead of the barbarous method of drying blood films at present almost exclusively in use, we cannot look for much certainty in the microscopical differentiation of very similar organisms. This must be supplemented by observing the differences in their pathogenic action.

We must not forget, either, the variability of organisms in the same species of animals in the same country, and their still greater variability in different animals in the same, and in other countries; and we must remember the great differences in the physical conditions of an organism which are due to environment. So I would not insist, with our present knowledge, too strongly upon the differences in form in the trypanosomata mentioned above, under the different conditions which have been described. But if preparations are made under the same conditions, and with the same care, it can be seen that the trypanosomata from the cases in which paralysis has occurred (in which very few are found in the blood, the greater number being in the central nervous system) are short and thick, with the flagellum extending very little beyond the body of the organism; they contain a varying number of vacuoles, and sometimes many granules; they move slowly, even when the smear of cord or brain is diluted with normal citrate solution (fig. 3). On the other hand, those found in the generalised cases (the Gambia Fever type) are long, with a long flagellum, and are not vacuolated, and move quickly (fig. 4). I have not found the other form in any of these cases, even when the animal has been long dying. This short, badly staining, vacuolated form is probably due to some condition of environment about which we know nothing, which produces not only changes in the morphology of the organism, but also in its pathogenic effects, for, from the above experiments, it can be seen that the inoculation of these short forms may, in the first or subsequent passage, give rise to the long form in the inoculated animal, with quite different symptoms (*cf.* figs. 3 and 4: fig. 3 photographed from a smear of spinal cord of Rat 66 *b*, and fig. 4 from a blood film of Rat 69, which was inoculated from Rat 66 *b*).

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* See Note on methods.

5. *Pathological Anatomy.*

The examination of these two series of rats has shown that the pathological effects are fairly uniform in each of the two types of cases.

In those animals in which paralysis occurred, the principal lesions were found in the spinal cord. There was invariably congestion of the vessels, and in eight cases well marked hæmorrhages around and in the substance of the cord. There was the characteristic exudation around the blood-vessels of mononuclear cells, and of plasma cells, such as Dr. Mott has described in the brain in cases of Sleeping Sickness; and in all the cases a varying amount of glia proliferation (*vide* figs. 1 and 2). The nerve-cells in the affected areas of the cord were degenerated in varying degrees up to the disappearance of the processes, and even of the nucleus with vacuolation of the protoplasm.

The brain showed much less change, and only in the chronic cases was any exudation found around the vessels. Small hæmorrhages in the meninges were seen in five cases.

Trypanosomata, mostly in stages of degeneration, were found scattered through the substance of the cord and brain in varying numbers; none were found in the vessels, which were generally distended with blood.

The other organs showed very little change; they were anæmic and firm; the spleen was not enlarged in the chronic cases with marked paralysis, and was only slightly enlarged in the transitional cases. There was no enlargement of the superficial or deep lymphatic glands, and the eyes and genitals were normal.

In the cases in which the trypanosomata were plenteous in the blood, and which had no paralytic symptoms (the Gambia Fever type), the brains and cords were very congested, and the blood in the distended vessels contained large numbers of trypanosomata. None were seen in the substance of the brain or cord. In six cases small hæmorrhages were found on the meninges. There was no degeneration of the brains or cords.

The superficial lymphatic glands were often enlarged. The abdominal glands were always enlarged, and some of both superficial and deep glands were reddish to brown in colour. The enlarged glands showed changes of a hyperplastic nature, and the reddish ones showed sinus formation, extreme congestion, and often hæmorrhages.

The lungs were always very congested, with sometimes subserous hæmorrhages in the lower lobes. Small hæmorrhages were found in 61 cases in the pleuræ.

The livers were always extremely congested, and were very soft in texture, and showed extensive parenchymatous degeneration, and often fatty infiltration and degeneration. The vessels were very dilated and distended, and often showed proliferation of their endothelium.

The stomach in 13 cases was ulcerated ; generally one small ulcer filled with clot was found, but sometimes as many as three or four. They were always found on the greater curvature. In two cases death occurred from perforation.

There was generally a diffused congestion of the lining membrane of the intestines, and on the peritoneal surface ecchymoses dotted about, either singly or in groups, were often found. The kidneys were congested, and had always macro- or micro-scopical hæmorrhages.

The spleen in every case was enormously enlarged, with great congestion of the pulp, and considerable hyperplasia of the follicles. There was generally proliferation of the endothelium of the distended vessels, and a great deal of phagocytosis of red blood corpuscles and trypanosomata.

The bone marrow (femur) was profoundly altered, having the appearance of red marrow. It was very cellular, with scarcely any fat, and there was always considerable phagocytosis, and many nucleated red corpuscles.

6. *Conclusions.*

From the above summary of 211 experiments, extending over a period of nearly three years, it will be obvious that the tentative deductions which I made in my Preliminary Note from the few experiments therein recorded, that Gambia Fever and Sleeping Sickness are two distinct diseases, cannot be maintained.

This extended series of experiments and observations go to show that each of these two strains of trypanosomata has produced two different effects in the same class of animals, under conditions of which we at present know nothing; and that these effects are alike for the two organisms; and that the trypanosomata found in these two types of disease are one and the same organism, modified by passage from man through monkeys to rats, and perhaps in the strains I have used, by transplantation into animals of, and in, another country.

NOTE ON METHODS.

With regard to the mention made in Section 4 of methods, the following, which has been used by the writer for some years, gives uniformly good and, as he believes, accurate results. The specimen is never allowed to dry, and there is no shrinkage of the cells, and the finest cytological details can be observed.

1. Expose a cover-glass to the vapour of

Osmic acid, 1 per cent.	1 c.c.
Glacial acetic acid	3—5 drops

for 2 minutes.

(This can be most conveniently effected by using a block-dish covered by a thin glass having a hole in it rather smaller than the cover-glass used.)

2. Place a drop of fresh blood in one corner of the cover-glass, and expose again to the vapour for 30 seconds.

3. Spread the film carefully, and expose again for 15 to 30 seconds to the vapour until the surface appears no longer moist. (The film will not be really dry, and can be easily smeared off the glass with the finger : a really dry film will be much lighter in colour, and cannot be rubbed off with the finger.)

4. Place the cover-glass in absolute alcohol for 10 minutes.

5. Place the cover-glass in a faintly rose-coloured solution of permanganate of potash for 1 minute. (Two or three drops of a 1-per-cent. solution to 50 c.c. of water.)

6. Wash in water for 5 minutes.

7. Stain in a modified Romanowsky's stain, made by mixing just before use—

Azur I, 1 per cent.	1 c.c.
Eosin, B.A., 1—1000	2 „
Water..	8 „

for 15—30 minutes.

8. Wash.
9. Differentiate in orange-tannin, 30 seconds.
10. Wash well and drain.
11. Absolute alcohol for a few seconds.
12. Alcohol—xylol (in proportion of 2 : 3), two or three changes.
13. Xylol, and mount.

Instead of 7—13, any other method of staining can be used, according to what structures it is desired particularly to show.

DESCRIPTION OF PLATE.

Fig. 1.—Section of half of spinal cord of rat which had been paralysed for 2 months before death. ×40. Shows meningitis, the exudation around the vessels, and a large area of degeneration.

Fig. 2.—Longitudinal section of vessel in spinal cord of rat which had been paralysed for 6 weeks before death. ×220. Shows the cellular exudation around the vessel, and on the right an area of degeneration.

Fig. 3.—Trypanosomata from a mash of spinal cord of rat, from which cord fig. 1 was photographed. ×1000. Stumpy forms, with very short flagella, staining badly, with very little differentiation.

Fig. 4.—Trypanosomata in blood of rat which was inoculated with the mash of spinal cord from which figs. 1 and 3 were photographed. Long, well-differentiated, easily staining forms. This rat was not paralysed.

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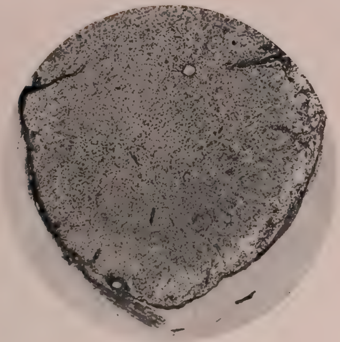

Fig. 1. × 40.

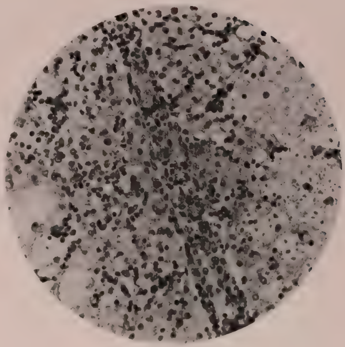

Fig. 2. × 220.

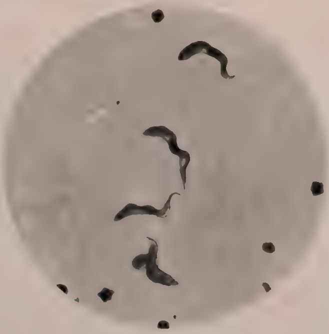

Fig. 3. × 1000.

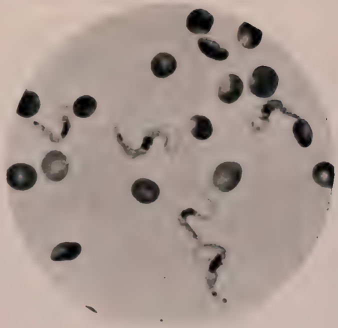

Fig. 4. × 1000.

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A Preliminary Summary of the Results of the Experimental Treatment of Trypanosomiasis in Rats.

By H. G. PLIMMER, F.L.S., and J. D. THOMSON, M.B., C.M.

(Communicated by Sir Ray Lankester, K.C.B., F.R.S. Received July 20, 1907.)

The experiments of which the following note is an abstract have been undertaken under the direction of the Tropical Diseases Committee of the Royal Society.

The strains of trypanosomes which have been used in these experiments are a Nagana from the original strain brought to England, and a Surra from Professor Lingard in India. The Nagana strain kills rats in an average time of 5·5 days, and the Surra strain in 6·9 days.

Chinolin Compounds.

The knowledge of the action of quinine in malaria suggested the trial of various chinolin compounds. As is well known, quinine itself has no influence in trypanosomiasis. Cyanin was the substance first tried, as this in extremely dilute solutions (1—10,000) is very poisonous to trypanosomes outside the body. A series of Nagana and Surra rats were given doses of from ½ to 1 milligramme (which is a poisonous dose for small rats), but all of these died about the same time as the controls, and in every case the trypanosomes had increased in the usual manner, so that at death the blood was as swarming with trypanosomes as if no treatment had been used. Messrs. Bayer and Co., of Elberfeld, kindly placed at our disposal nine chinolin and chinaldin compounds and six "cyanin farbstoffe," but none of these produced any effect on the development or course of the diseases.

Dichlorobenzidine + Acid H.

This substance, which was first used by Nicolle in trypanosomiasis of mice, was found, when used both on mice and rats, to lengthen the course of the diseases, but in no case was the extension of the course of the disease very marked. Nagana mice, after one dose of 1 c.c. of a 1-per-cent. solution, lived from 8 to 11 days, and Surra mice from 10 to 16 days, one living as long as 29 days; in Nagana rats the disease was prolonged to an average time of 14 days, and in Surra to an average of 16 days. In all the animals treated with this substance the spleen and liver were very much enlarged, and living trypanosomes were found *post mortem*, showing that the substance had not practically influenced the pathological processes. In no case was the substance completely absorbed.

Trypanroth.

This was discovered and first used by Ehrlich. When used alone it lengthened the course of the disease in both Nagana and Surra to about 14 days, but in all cases the spleen was found to be as much enlarged as in the control, and living trypanosomes were invariably found *post mortem.*

Arsenious Acid.

This was used at first simply dissolved in water, and later, in order to try to avoid the irritation and sloughing invariably produced, in a solution slightly over-neutralised with carbonate of soda. When injected before the fourth day of Nagana and Surra, arsenious acid caused the disappearance of the trypanosomes from the blood, but they invariably recurred before death. It was given in doses of 1 milligramme, and one rat lived as long as 26 days, two to 16 days, and one to 17 days, each of these rats having taken 6 milligrammes altogether. A very considerable effect was produced on the vitality of the trypanosomes, as a rat inoculated from the bone-marrow of one of the 16-day rats did not show trypanosomes in the blood until the ninth day after inoculation. Whether neutralised or not, sores were produced at the points of injection. The spleen was found *post mortem* to be about as large as that of the controls.

Atoxyl.

This substance, which was first used by Thomas and Breinl, has been found by Ehrlich and Bertheim to be the sodium salt of para-amido-phenyl-arsenic acid, with the formula

$$NH_2(C_6H_4)AsO.ONa.OH,3H_2O,$$

and Moore, Nierenstein, and Todd* have confirmed this view of its composition, which differs from that originally published, which stated it to be an anilide of metarsenious acid.

This is the most important substance, so far discovered, in relation to the treatment of trypanosomiasis. In all cases of Nagana and Surra (with the exception of the atoxyl-proof cases mentioned below), atoxyl causes the entire disappearance of the trypanosomes from the blood, so that rats inoculated with the blood when it was microscopically free from parasites failed to take the disease; but the trypanosomes, in our experience, have invariably recurred, and death was only delayed for a period varying with the dose and with the time of commencement of the treatment. When the dose was sufficient, *e.g.*, 1 to 1·5 c.c. of a 5-per-cent. solution in three to five doses

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* "On the Treatment of Trypanosomiasis by Atoxyl . . . . followed by a Mercuric Salt," etc., 'Biochemical Journal,' vol. 2, Nos. 5 and 6.

(a dose being given every, or every other, day), even after as many as four recurrences the spleen was generally found *post mortem* to be very little or at most moderately enlarged. A too large dose apparently produced wasting, and a bad condition of the hair, and, we think, a quicker return of the trypanosomes.

When atoxyl is given more continuously or too freely than is required, in cases in which there have been many recurrences, and probably under some other conditions of which we are ignorant, in a certain small proportion of rats so treated a race of trypanosomes is produced which entirely resists atoxyl, and continues to develop and multiply in spite of continued exhibition of the drug. This strain, when inoculated into fresh rats, retains its resistance to atoxyl. Ehrlich, who has produced such a strain in mice, calls them "atoxyl-fest," and we have obtained this atoxyl-proof variety of trypanosome in rats, both in Nagana and Surra, of which mention will be made later.

1. *Monophenylarsenic acid.*
2. *Nitrophenylarsenic acid.*
3. *Paratolylarsenic acid.*

These three bodies were sent for experimental purposes to Professor Cushny, F.R.S., by Professor Michaelis, who discovered them. They are of interest in connection with the treatment of trypanosomiasis, since atoxyl is an amido derivative of phenylarsenic acid, which was also discovered by Michaelis. They are extremely poisonous substances, and were given in doses of 1 c.c. of a solution of 1/400.

1. Monophenylarsenic acid.—This, in common with other arsenic compounds, notably affected the development of the trypanosomes. In doses of 1 c.c. of 1/400, repeated once or twice in untreated animals, it diminished greatly the number of trypanosomes; and in three recurrent cases, after initial treatment with atoxyl, it caused the trypanosomes to disappear, but it did not save any animal; and it is, as are all these three compounds, poisonous in effective doses.

2. Nitrophenylarsenic acid.—In untreated animals, and in two recurrent cases after atoxyl, this caused a temporary disappearance of the trypanosomes; in a case of second recurrence after atoxyl, it produced no effect on the trypanosomes. It is the most poisonous of the three.

3. Paratolylarsenic acid.—This caused a more effective disappearance of the trypanosomes than the other two compounds, and it was better borne. One Nagana rat, treated only with it from the beginning, lived for 25 days, having had, however, three recurrences during this period. At each recurrence the trypanosomes disappeared after two doses of 1 c.c. of 1/400. In recurrent

cases after atoxyl a temporary disappearance of the trypanosomes was also effected.

Other Arsenic Compounds.

Arrhenal (di-methyl sodium arseniate) and sodium cacodylate were also tried, but were found to have no effect whatever on the course of the disease, nor upon the development of the trypanosomes either in first infections or in recurrences.

Other Bodies.

Fluorescein was tried without any effect; and also sodium cinnamate, on account of its reputed property of producing leucocytosis, which might have been useful from the phagocytic standpoint. But it failed to have any effect in these diseases.

Treatment with two or more Drugs.

The deduction from the above-mentioned experiments with single bodies is that, at the present time, there is no substance known which alone will cause a permanent disappearance of the trypanosomes from the affected animal, *i.e.*, effect a cure; so that experiments have been carried out to see whether any combination of bodies would produce the desired effect. The compounds of mercury seemed from their known properties to be the most promising, and the results of experiments in this direction, lately published by Drs. Moore, Nierenstein, and Todd, are most encouraging. We have not used the form of mercury (liq. hydrarg. perchlor.) with which these good results have been obtained, but we have used the lactate, sozoiodol, and succinimide, and also Donovan's Solution (liq. arsen. et hydrarg. iodid.). Of these the succinimide appears to us to be the best, as it is unirritating, and will apparently mix with atoxyl without change—at any rate it does not interfere with the action of the atoxyl; the lactate is very irritating, even in doses of one-third of a minim of the ordinary hypodermic solution, and it is not so effective as the succinimide; the sozoiodol appears to come between these two in point of efficiency. But in our experience there is a great difference in the individual equation of rats with regard to dosage and poisonous effects, which the consideration of relative weight does not help us to gauge, so that there has been difficulty in ensuring the best method of giving these drugs, and of determining their appropriate quantity. The duration of the disease has been very greatly prolonged in most cases, and in some we have confidence that a cure has been effected.

Of all the substances tried, atoxyl has had by far the most favourable action, and it is easily tolerated, and produces no irritation. But, as mentioned before, it has no permanent effect; it causes the disappearance of the trypanosomes from the blood, even when in very large numbers, but there remain behind certain forms of the parasites which can resist it, and which after an interval can reproduce the general infection with the indifferent forms. These recurrences can take place, in our experience, up to as many as seven times. The resistant forms appear to be located in the intermediate periods in the bone-marrow, but there is some doubt about this yet, as in some cases we have failed to produce an infection, even with a quantity of the marrow. It is probable also that some of these resistant forms may be found in the glands, and observations are being carried out on these points. But when an infection is produced from the marrow of these atoxyl-treated rats, the incubation period is very much prolonged, and it may be as long as nine days before trypanosomes can be found in the blood of the inoculated animals.

Atoxyl appears to act not by virtue of the arsenic in it alone, but its effects are probably due to this in combination with some other of its constituents, as its action is much more rapid and complete than that of any other of the simpler arsenic compounds which we have tried.

The following Tables I and II give the results of treatment with atoxyl and succinimide of mercury: this latter has been given either with the atoxyl or immediately after it, the best method being, we think, to give two or three doses of atoxyl and to give a dose of succinimide of mercury at the same time as the second and third doses, with perhaps one after. Some of our rats have had too much, and we are now giving less with better effect. The succinimide has the great advantage of being unirritating to the tissues, and it will mix with atoxyl without precipitation, and without, at any rate, interfering with the action of the latter.

Table I.—Nagana Rats treated with Atoxyl and Succinimide of Mercury. Average duration of untreated disease, 5·5 days.

No.	Weight.	Quantity of 5 p. c. atoxyl.	Quantity of succinimide of mercury.	Recurrences.	Lived.	
	grammes.	c.c.	milligrammes.		days.	
1	175	2·4	1·5	2	23	Had young on 20th day.
2	210	2·85	1·75	1	30	Did not die of disease.
3	250	2·1	2·5	1	28	Kidneys degenerated.
4	200	2·1	2·75	0	—	*Living* 143 *days.*
5	175	2·0	0·75	1	27	Did not die of disease.
6	150	1·8	1·25	0	—	*Living* 140 *days.*
7	200	1·8	1·25	0	—	*Living* 136 *days.*
8	150	2·75	2·0	6	79	
9	175	2·6	1·25	0	22	Succinimide given too long after the atoxyl.
10	175	3·0	1·25	1	—	*Living* 92 *days.*
11	125	1·8	0·8	0	38	
12	200	1·2	0·75	1	—	*Living* 85 *days.*
13	160	2·8	0·75	3	36	
14	210	4·5	1·75	4	59	
15	175	1·75	0·5	0	—	*Living* 78 *days.*
16	190	1·4	0·25	0	—	*Living* 75 *days.*
17	200	4·1	1·75	4	42	Trypanosomes plentiful at time of death, probably atoxyl-proof.
18	175	6·35	2·0	7	107	This rat had 5 minims. of Donovan's solution on the initial appearance of trypanosomes, but, as it had no effect, atoxyl, and subsequently succinimide, were given.
19	160	4·1	0·75	3	61	
20	150	3·7	0·5	3	44	Given paratolylarsenic acid after third recurrence, which killed the rat.
21	150	0·7	0·75	0	—	*Living* 37 *days.*

Table II.—Surra Rats treated with Atoxyl and Succinimide of Mercury. Average duration of untreated disease, 6·9 days.

No.	Weight.	Quantity of 5 p. c. atoxyl.	Quantity of succinimide of mercury.	Recurrences.	Lived.	
	grammes.	c.c.	milligrammes.		days.	
1	175	1·5	2·25	0	43	Did not die of disease.
2	175	1·5	2·25	0	68	Did not die of disease.
3	150	1·5	1·25	0	44	Did not die of disease.
4	200	5·1	2·75	3	63	
5	125	1·8	1·5	0	—	*Living* 137 *days.*
6	125	1·8	0·5	0	120	Did not die of disease.
7	155	1·8	0·75	1	24	
8	175	2·45	1·0	1	23	This quantity of succinimide produced an acute nephritis in this rat, with necrosis of the epithelium of the convoluted tubes, and many hæmorrhages.
9	160	3·3	1·0	1	—	*Living* 91 *days.*
10	150	3·0	0·75	4	55	
11	150	2·4	0·75	2	32	
12	160	2·1	0·37	1	22	Treatment suspended on 10th day.
13	160	1·8	0·25	2	32	Did not die of disease.
14	145	2·1	0·75	0	—	*Living* 42 *days.*
15	120	1·9	0·5	0	—	*Living* 39 *days.*
16	125	3·0	1·0	2	34	This rat had 2 c.c. iodipin on 3rd day.
17	200	4·1	1·75	4	42	
18	145	1·05	0·25	0	—	*Living* 42 *days.*
19	120	0·95	0·5	0	—	*Living* 39 *days.*
20	145	1·05	0·65	0	—	*Living* 33 *days.*
21	125	2·1	0·75	3	54	This rat had at the outset 8 mgms. in all of arsenious acid, the atoxyl being given on the first and subsequent recurrences.
22	155	1·5	1·0	1	32	This rat had also initial treatment with arsenious acid, of which it had 6 mgms. in all, the atoxyl being given on the first recurrence. The rat did not die of the disease, but from injury during pregnancy.

(See also No. 8, Table III.)

Table III gives the results of treatment with atoxyl and mercury sozoiodol, from which it will be seen that the results are not so good as those with succinimide of mercury. Mercury sozoiodol will not mix with atoxyl, a dense precipitate being formed when the solutions are mixed. The solution used was the ordinary hypodermic solution (1·5 per cent.) prepared by Martindale.

Table III.—Nagana and Surra Rats treated with Atoxyl and Mercury Sozoiodol. Average duration of untreated diseases 5·5 and 6·9 days respectively.

Disease.	No.	Weight.	Quantity of 5 p. c. atoxyl.	Quantity of mercury sozoiodol.	Recurrences.	Lived.	
		grammes.	c.c.	minims.		days.	
Nagana	1	200	1·35	1	1	24	
	2	150	1·4	1	1	26	
	3	145	1·8	1	0	—	*Living* 108 *days.*
	4	155	2·4	1⅓	1	26	
Surra …	5	275	1·8	1⅓	0	—	*Living* 122 *days.*
	6	150	1·8	1	0	35	Did not die of disease.
	7	315	5·7	1⅔	4	59	
	8	150	6·8	1⅔	7	81	After second recurrence with mercury sozoiodol, succinimide of mercury was substituted, of which 0·75 mgm. was given in all.
	9	150	2·7	⅔	1	33	After first recurrence, this rat was given succinimide of mercury, of which it had 1 mgm. in all.

Table IV gives the results of treatment with atoxyl and lactate of mercury, which are less good than those with succinimide and soziodolate of mercury. This drug has the disadvantage of being very irritating to the tissues, and also of forming a precipitate when mixed with atoxyl. The solution used was the ordinary hypodermic solution (2·5 per cent.) prepared by Martindale.

Table IV.—Nagana and Surra Rats treated with Atoxyl and Lactate of Mercury Average duration of untreated diseases, 5·5 and 6·9 days respectively.

Disease.	No.	Weight.	Quantity of 5 p. c. atoxyl.	Quantity of lactate of mercury.	Recurrences.	Lived.	
		grammes.	c.c.	minims.		days.	
Nagana	1	175	1·85	1	1	25	
	2	150	2·1	1	1	26	
	3	150	1·5	1	0	23	
	4	175	3·65	1	1	31	This rat had 0·5 mgm. of succinimide of mercury after first recurrence.
Surra	5	200	3·9	1	1	32	
	6	275	3·45	1	1	30	
	7	175	1·2	1	0	21	Did not die of disease.
	8	315	3·6	1	1	24	
	9	125	3·0	⅔	2	35	This rat had 1 mgm. of succinimide of mercury after first recurrence.

Table V gives the results of a few experiments made with atoxyl and Donovan's solution (liq. arsenic et hydrarg. iodid.) which are somewhat encouraging. During the last month a fresh and more extensive series of experiments have been started. The poisonous effects of Donovan's solution show themselves in the intestines, which become intensely inflamed, whereas the effects of too large doses of succinimide of mercury show themselves principally in the kidney, in the form of an acute inflammation going on to necroses with multiple hæmorrhages.

Table V.—Nagana and Surra Rats treated with Atoxyl and Donovan's Solution. Average duration of untreated diseases, 5·5 and 6·9 days respectively.

Disease.	No.	Weight.	Quantity of 5 p. c. atoxyl.	Quantity of Donovan's solution.	Recurrences.	Lived.	
		grammes.	c.c.	minims.		days.	
Nagana	1	130	1·8	5	2	38	This rat had 2·5 c.c. 1/400 monophenylarsenic acid after first recurrence; this removed the trypanosomes from the blood, but a second recurrence took place 11 days later.
Surra	2	175	3·7	17	4	—	*Living* 67 *days.*
	3	150	2·4	15	3	72	
	4	200	2·25	4 + 0·5 c.c.	1	28	The 0·5 c.c. of Donovan's solution was given to test dosage.[*] Next day the rat became very ill, and died two days after with acute enteritis. No kidney lesion.
	5	190	2·25	5	1	23	
	6	300	1·0	8	0	—	*Living* 36 *days.*

[*] See paper by Drs. Moore, Nierenstein, and Todd, before referred to.

Table VI gives the results of a series of experiments made with atoxyl and iodipin. This latter is a solution, or mixture, of iodine in oil of sesame, made by Merck, who kindly placed some at our disposal, and it is stated to be unirritating, and it can be given in large doses with safety. The results are sufficiently encouraging to suggest a further trial of this combination. The oil takes a very long time to get absorbed; it becomes colourless from absorption of the iodine, and appears to cause no irritation in the tissues.

Table VI.—Nagana and Surra Rats treated with Atoxyl and Iodipin. Average duration of untreated disease, 5·5 and 6·9 days respectively.

Disease.	No.	Weight.	Quantity of 5 p. c. atoxyl.	Quantity of iodipin.	Recurrences.	Lived.	
		grammes.	c.c.	c.c.		days.	
Nagana	1	275	4·9	7·5	2	35	
	2	150	5·4	9·5	5	71	
	3	150	1·8	6·0	0	26	
	4	195	1·5	2·0	0	112	Did not die of disease.
	5	175	2·1	6·0	2	23	Did not die of disease.
	6	275	4·0	7·5	0	29	Did not die of disease.
	7	175	4·5	7·0	3	44	
Surra...	8	175	1·9	4·0	0	57	Had eight young towards end of treatment.
	9	195	1·5	2·0	0	—	*Living* 132 *days.*
	10	195	1·5	2·0	0	—	*Living* 132 *days.*
	11	143	6·6	12·0	2	44	
	12	250	4·1	4·0	1	29	Did not die of disease.
	13	250	4·0	4·0	2	24	
	14	215	4·5	6·5	0	—	*Living* 103 *days.*
	15	165	2·9	4·5	2	33	
	16	150	3·3	6·0	3	31	Did not die of disease.
	17	150	1·8	2·5	1	20	Did not die of disease.

The Production of an Atoxyl-proof Race of Trypanosomes in Rats.

This phenomenon is of great biological and therapeutical interest, and needs much further research yet, both for its explanation, and its bearing on treatment.

In certain rats, after treatment with atoxyl, a number of recurrences may take place—we have seen as many as seven; these recurrences at first yield to treatment with atoxyl, but as the case progresses towards its end, the recurrences occur at shorter and shorter intervals, until one comes to a time when they can be no longer affected by atoxyl. The trypanosomes in this case have become atoxyl-proof. Ehrlich succeeded in producing an atoxyl-proof strain of Nagana in mice, and we have succeeded in producing atoxyl-proof strains of both Nagana and Surra in rats. One point of importance biologically is that these atoxyl-proof trypanosomes do not lose their resistance to atoxyl on transference into other rats, and we have carried this strain on in Nagana through a series of eight successive rats, and in Surra through a series of six rats without any loss of their atoxyl-proof properties. When treated with atoxyl, even in large doses, multiplication goes on just as rapidly as in untreated animals, and the rats die at the usual time. Our cases have been given atoxyl to test them, and have been then treated with various substances, as set forth in the table below. Of these substances, trypanroth and some form of mercury have given the best results. In thinking of human trypanosomiasis in this connection, the danger of the production of an atoxyl-proof strain will be at once apparent: so that atoxyl should be given to human beings with the greatest care and forethought.

The following Table VII summarises the few results we have obtained so far. Rat No. 1 was inoculated from a Nagana rat which had become uninfluenced by atoxyl after the third recurrence, before which large and repeated doses had been given: and Rat No. 11 was inoculated from a Surra rat in a similar condition.

Table VII.—Summary of Results obtained with Atoxyl-proof Strains of Nagana and Surra in Rats.

Disease.	No.	Weight.	Trypanroth 1 per cent.	Succinimide of mercury.	Donovan's solution.	Dichloro-benzidine + acid H 1 per cent.	Paratolyl-arsenic acid 1/400.	Lived.	
		grammes.	c.c.	milligrammes.	minims.	c.c.	c.c.	days.	
Nagana	1	130	—	—	—	—	—	6	Treated only with atoxyl.
	2	150	—	—	—	—	—	6	Treated only with atoxyl.
	3	140	—	0·25	—	—	—	6	Did not influence the course of the disease.
	4	250	5	0·5	—	—	2	17	Trypanroth caused a disappearance of the trypanosomes for 6 days: paratolylarsenic acid did not affect them.
	5	200	6	0·5	—	—	2	11	Neither trypanroth nor paratolylarsenic acid did more than restrain for a few days the multiplication of the trypanosomes in this case.
	6	175	—	0·75	—	6	1	10	Dichloro-benzidine + acid H caused the trypanosomes to disappear for 3 days.
	7	150	—	—	—	—	2	6	Trypanosomes were not affected.
	8	125	4	—	8	—	—	14	Trypanosomes driven out of the blood for 5 days.
	9	175	6	—	6	—	—	12	Trypanosomes driven out of the blood for 3 days.
	10	225	3	—	4	—	—	10	Trypanosomes only disappeared the day before death.
	11	125	—	—	—	—	—	3	Did not die of disease.
Surra ...	12	150	3	—	—	—	—	5	
	13	125	3	—	—	—	—	6	
	14	105	3	0·62	—	—	—	10	

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*Further Results of the Experimental Treatment of Trypanosomiasis in Rats; being a Progress Report of a Committee of the Royal Society.**

By H. G. Plimmer, F.L.S., and J. D. Thomson, M.B., C.M.

(Communicated by Sir Ray Lankester, K.C.B., F.R.S., Chairman of the Tropical Diseases Committee. Received October 28,—Read November 7, 1907.)

[Plate 1.]

The following results are a continuation of the work already described, and the experiments have been carried out upon rats inoculated with the same strains of Nagana and Surra:—

Condition of the Animals living at the Date of the Completion of the Tables in the former Paper.

Table I.—Nagana Rats treated with Atoxyl and Succinimide of Mercury.

No. 4 is still living and well 229 days after inoculation.
„ 7 „ „ 222 „ „
„ 10 „ „ 178 „ „
„ 15 „ „ 164 „ „
„ 21 „ „ 63 „ „
„ 6 died on the 214th day after inoculation.
„ 12 „ 110th „
„ 16 „ 81st „

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* This Committee, which planned and supervised the investigations, was appointed by the Tropical Diseases Committee for the purposes of this experimental enquiry, and consists of the following members: Professor J. Rose Bradford, Colonel Bruce, Professor Cushny, and Mr. H. G. Plimmer. A preliminary summary of the results of the enquiry

In these rats the livers were found to be pale and fatty, and the kidneys degenerated: these were pale and fatty, with fibrous streaks, and the urine of Nos. 12 and 16, found in the bladder *post mortem*, contained albumen. They did not die of the disease, as no trace of trypanosomes could be found in either of them, but of the degenerative changes mentioned.

Table II.—Surra rats treated with Atoxyl and Succinimide of Mercury.

No. 5 died on the 206th day after inoculation, of pneumonia.
" 9 " 169th " " "
" 14 " 61st " from an unnoticed recurrence.
" 15 " 59th " from paralysis after three recurrences.
" 18 " 61st " .
" 19 " 59th " .
" 20 " 57th " after five recurrences, becoming finally axotyl-proof.

In Nos. 5 and 9 there was also evidence of fatty and fibrous degeneration of the kidneys.

Of these rats only in Nos. 14 and 20 was there any evidence that they died of the disease.

Table III.—Rats treated with Atoxyl and Mercury Sozoiodol.

No. 5 is still living and well, 208 days after inoculation.
" 3 died on the 181st day after inoculation.

(The kidneys and liver of No. 3 were very fatty.)

Table V.—Rats treated with Atoxyl and Donovan's Solution.

No. 2 died on the 70th day after inoculation.
" 6 " 121st " "

(The kidneys of No. 2 were degenerated, those of No. 6 markedly so, being pale in colour with yellow streaks, and very friable; there was albumen in the urine.)

Table VI.—Rats treated with Atoxyl and Iodipin.

No. 9 is still living and well 218 days after inoculation.
" 10 died on the 141st day after inoculation.
" 14 " 178th "

(No. 14 had also 1·2 milligrammes of succinimide of mercury. The kidneys of this rat were degenerated, and it had albumen in its urine.)

From the above list it will be seen that the principal pathological lesion in those rats which have been treated with atoxyl and some compound of mercury and have lived for a very long time after inoculation, being, we think, cured of the disease, is a degeneration of the kidneys; and in most of

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has already appeared in the 'Proceedings of the Royal Society' (B, vol. 79, 1907, pp. 505—516). By the courtesy of the governing body of the Lister Institute the investigations have been carried on in the laboratories of that institution.

these rats this was the only lesion found *post mortem.* This will be referred to later in mentioning further treatment with mercury.

Atoxyl and Calomel.

Twelve rats have been treated with atoxyl (three to five doses) and then with subcutaneous and intramuscular injections of calomel, in doses of 1 minim of Surgeon-Major Lambkin's formula. It is difficult in rats to make the injection into the muscles, and in all cases necrosis occurred at the site of the injection; no better result was attained than by treatment with atoxyl alone, with subsequent recurrences and death.

Atoxyl and Succinimide of Mercury.

Further experiments have been made with this combination, in which the dose of the mercury salt has been increased up to 1 milligramme. The 12 rats of this series are all dead, and showed acute kidney changes: inflammation, going on to necrosis of the epithelium, multiple hæmorrhages, etc.

Atoxyl and Donovan's Solution.

Nine further experiments have been made with this combination, also in larger doses; but these larger doses have been invariably fatal, with lesions both of the intestines and kidneys. The doses were arranged upon the basis of the doses recorded, with such good results, by Drs. Moore, Nierenstein, and Todd;* but one of us has received a letter, since the experiments were completed, from Dr. Nierenstein, stating that the Donovan's solution used in their experiments was diluted with an equal part of water.

Atoxyl and Liq. Hydrarg. Perchlor.

A series of 12 rats was treated with this combination on the lines laid down in the paper above referred to, by Drs. Moore, Nierenstein, and Todd. The results obtained by them gave much hope that this combination would be especially useful. But we have not been able to get such good results, Out of 12 rats so treated only one is alive at the 97th day, the others having died with acute renal lesions. Of course, really comparative results are always difficult to obtain, and in rats the individual equation, with regard to dosage, and to resistance both to drugs and to disease, is a very varying one. We have, for instance, found, with the rats we have used, that the white ones are more susceptible both to diseases other than trypanosomiasis and to drugs than the black and white ones are; and we find the grey are the least susceptible.

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* "On the Treatment of Trypanosomiasis by Atoxyl . . . . . followed by a Mercurial Salt, etc.," 'Biochemical Journal,' vol. 2, Nos. 5 and 6.

From the above, considering both those experiments recorded in our former paper which have since ended fatally, and the more recent and—as regards dosage—bolder experiments, we are forced to the conclusion that, in small animals at any rate, mercury has not in our hands given altogether satisfactory results. Perhaps it may be a question of dosage; we have, however, tried to enlarge the range of dosage, as far as possible, from homœopathic doses to large ones, without attaining a large percentage of cures. If the dose of mercury be sufficient to aid the atoxyl, as in the cases brought forward from our last paper, we have found, in those cases which have died, chronic kidney, and in a less degree liver, lesions, which seem to be the late result of those more acute changes which we have found in those animals which have died earlier, either from disproportionate dosage or from some want of resistance to the drug.

Perhaps, in dealing with a more chronic trypanosome disease such as Sleeping Sickness in man, the results would be more favourable. We have, for instance, two Sleeping Sickness rats, inoculated on April 15, which have been treated with quite small doses of atoxyl and succinimide of mercury, and n which no trypanosomes have been found since May 4; they appear to be quite well. We have also another Sleeping Sickness rat, inoculated on May 6, which has been treated only with atoxyl, and in which no trypanosomes have been found since May 28. But in the more acute forms of trypanosomiasis, such as Nagana and Surra, the method of treatment by atoxyl and some form of mercury has, in our experience, almost invariably led to degenerative lesions, principally in the kidneys, which has been a cause of death long after any trace of trypanosomes could be found, when we have believed that the animal has been quite cured of the initial disease.

Tiodine.

A few rats were treated with this substance, which is thiosinaminethyliodide ($C_6SN_2H_{13}I$). In doses of 10 minims it is immediately fatal to rats, although it is stated to be non-poisonous to man, and in doses of 5 minims caused death within 24 hours; in smaller doses, alone or in combination with atoxyl, it had no influence on the disease.

Certain Antimony Compounds.

The treatment with arsenic compounds was, as has been stated, attended with only partial success. Professor Cushny, F.R.S., who has advised us on pharmacological matters throughout these investigations, sent us for trial a weak combination of glycine and antimony, attempts to form an antimony compound analogous to atoxyl having failed. When injected into inoculated rats, the antimony glycine was found to possess, in a less degree, the power of the antimony compounds described below; it reduced the number of trypanosomes, and caused their disappearance from the blood for a time, if they were not very numerous.

But the combination was difficult to make, varied in strength, and its solution was very dilute, and its use was abandoned when it was found that the other antimony compounds, described below, were so much more effectual.

Potassium Antimonyl Tartrate.

Potassium antimonyl tartrate (tartar emetic), which is easily soluble, was then tried. In doses of 1 c.c. of a 1 per cent. solution it proved fatal within 24 hours to four inoculated rats of weights varying from 190 to 225 grammes, but it was noticed that the trypanosomes had greatly diminished in number. It was also noticed that the rats appeared to be ill and faint for a short time after the injection: they were unsteady and sometimes rolled about. This was at the time attributed to the depressing effect of the potassium in the compound, and suggested the making and using of the substance described below, with which all of our experiments have so far been done. The question of the effect of this particular salt on the rats themselves will be mentioned later. Experiments are in progress for the purpose of comparing the actions of the potassium and sodium compounds: the latter would seem to possess some theoretical advantage, especially in treating larger animals, when the dose will have to be proportionally larger.

Sodium Antimonyl Tartrate.

Through the kindness of Dr. R. H. Aders Plimmer, of University College, we have been able to obtain and use this compound, which is the sodium salt corresponding to potassium antimonyl tartrate. He has prepared a quantity of the pure substance for us, in crystalline form, and has written the appended Note* upon its chemistry.

This substance in 1 per cent. solution is that which, of all the various bodies mentioned in these papers, including atoxyl, has the most marked and remarkable influence upon trypanosomes in the living body. Although our experiments with it are not many, nor of long duration, the results so far seemed sufficient to induce us to call the attention of other workers in this field to it.

The injection of this compound causes no pain, nor does it produce any inflammation of the tissues; and the results of the injection are very striking. The trypanosomes disappear with great rapidity from the blood, and in the majority of the cases treated so far there has been no recurrence; and inoculations made from animals which have been killed, or have died, have been invariably negative. It acts much more quickly than atoxyl, and its dose is very much smaller, and it does not produce any undesirable effects on the animals, and recurrences are very much less frequent than is the case when atoxyl has been used.

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* See Note at end of paper, p. 11.

We have used a 1 per cent. solution of the solid salt, but this is quickly invaded by moulds, and needs the addition of a crystal of thymol, or of 0·25 per cent. of formalin. The question of dosage is still under observation. We have tried many ways, and at present we are inclined to think that a full dose (*e.g.*, 0·5 c.c. of a 1 per cent. solution for a rat of 200 grammes or over) should be given when the trypanosomes are fairly plentiful in the blood, and then repeated at intervals of one, two, and three days, up to about four doses, and thereafter in weekly doses for a month. But we have good results in cases in which a dose has been given on four successive days, also when given every other day, and so on up to once every five days, without any recurrence up to as many as 52 days; but of two cases dosed at five-day intervals, one has recurred and one has not. As regards the quantity which can be taken, we have one rat of 130 grammes weight which has taken 0·5 c.c. of a 1 per cent. solution on the 3rd day (when trypanosomes were plentiful in the blood), and again on the 5th, 0·29 c.c. on the 6th and 7th days, and 0·25 c.c. on the 8th, 9th, 10th, 12th, 13th, 14th, 15th, 16th, 17th, and 19th days, and it is still living and well on the 43rd day, and has had no recurrence. When it is given in a full dose for the first time to rats whose blood is swarming with trypanosomes, the rat generally becomes very restless, and rolls about; its respirations become very quick, and it appears to be ill. These symptoms were noticed in the initial experiments with tartar emetic, and were attributed to the potassium contained in it, but we think that they are more probably due to the changes in the blood caused by the destruction or solution of the trypanosomes, which occurs very rapidly, as they do not occur after the second dose, when there are no trypanosomes. Recovery has, so far, always taken place from this condition, but it would seem advisable, in cases where the first dose is delayed until the blood is swarming with trypanosomes, to give the dose in two halves at intervals of a few hours.

The quickness of the action of sodium antimonyl tartrate is very remarkable. In one rat, whose blood was swarming with trypanosomes, a dose of 0·35 c.c. of a 1 per cent. solution caused their entire disappearance from the blood within half an hour; and in two other cases, in which the blood contained very large numbers of trypanosomes, after injection of 0·33 c.c. only a few could be found at the end of half an hour, and in one after an hour none could be found, and in the other only one in an ordinary blood preparation (see Plate 1). In these cases a few trypanosomes can sometimes be found in the liver, and these are extremely active and in no way inconvenienced by the drug; whether these are the forms which can persist, and need to be tired out by successive doses, we cannot say at present, but their extreme activity, when all the others have disappeared, is suggestive. We have up to the present not detected any morphological differences in them.

A striking instance of the power of this compound over trypanosomes is seen in the case of a guinea-pig which was inoculated with *Trypanosoma Gambiense* on April 9. From July 3 to September 16, trypanosomes were present in the blood, latterly in quantity, and the animal was dying on September 16; its eyelids were œdematous and nearly closed; it had œdema of the genitals and anus, and a discharge of bloody mucus from the rectum, and its hair was coming out in large patches. At this date—September 16—it was given 0·5 c.c. of a 1 per cent. solution; on the 17th the trypanosomes had entirely disappeared, and 0·75 c.c. was given; on the 19th the animal to all appearances was quite well, and on this day, and on 21st and 26th, 1 c.c. was given. The œdema disappeared and it continued to look well, and showed no more trypanosomes. It lived until October 14, when it died; *post mortem* the organs were congested and the kidneys were inflamed and the urine in the bladder contained albumen. The fact that the guinea-pig was moribund when the treatment was commenced may reasonably account for the pathological condition.

The following table shows the general results, as obtained so far, of the treatment with sodium antimonyl tartrate. Of the 39 rats enumerated in this table, the first 3 had been treated at first with antimony glycine; and of the 11 of the remaining 36 which have died, 6 did not die of the disease, and there remain alive and well 3 of 52 days, 1 of 49, 7 of 44, 8 of 43, 4 of 31, and 2 of 21; and of these 25, 23 have had no recurrence. It will be seen that 8 of the rats (4 Nagana and 4 Surra) have been treated with mercury in addition to the sodium antimonyl tartrate, in order to see if these obtained thereby any advantage over those not so treated. So far as we can tell at present, there seems to be no obvious advantage: one of the Nagana rats treated with liq. hydrarg. perchlor. has had three recurrences, and one Surra rat treated with succinimide of mercury has died after one recurrence.

Sodium Arsenyl Tartrate.

As the results with the sodium antimonyl tartrate were so definite, it seemed worth while to investigate the effect of the corresponding arsenic compound.

Table of Nagana and Surra Rats treated with Sodium Antimonyl Tartrate. Average duration of untreated diseases 5·5 and 6·9 days respectively.

Disease.	No.	Weight in grammes.	Total amount of 1 per cent. sodium antimonyl tartrate in c.c.	Result to Oct. 24.	Recurrences.	Remarks.
Nagana ...	1	150	6·2	Died on 49th day...	2	Antimony glycine had been given at first, sodium antimonyl tartrate on first recurrence.
,,	2	200	0·83	,, 25th ,, ...	1	,, ,, ,, No trypanosomes were found for 12 days before or at death.
,,	3	170	1·75	,, 35th ,, ...	1	Antimony glycine had been given at first, sodium antimonyl tartrate on first recurrence. No trypanosomes found for 10 days before, or at death, and a mouse inoculated with bone marrow did not take the disease.
,,	4	115	2·76	,, 38th ,, ...	1	No trypanosomes found for 16 days before, or at death. Rat became paralysed.
,,	5	175	1·35	,, 16th ,, ...	1	Probably too small a dose.
,,	6	125	1·0	,, 18th ,, ...	2	,, ,,
,,	7	200	3·0	*Living 52 days......*	0	
,,	8	150	2·5	,, *52* ,, ...	0	
,,	9	250	2·6	,, *52* ,, ...	0	
,,	10	215	3·5	,, *49* ,, ...	0	
,,	11	125	2·6	Died on 22nd day...	1	Died of septicæmia from sloughing tail, not of disease. No trypanosomes found.
,,	12	195	3·25	,, 19th ,, ...	0	,, ,, ,,
,,	13	175	1·85	,, 40th ,, ...	0	Died of septicæmia from an abdominal abscess, not of disease. No trypanosomes found.
,,	14	110	1·9	*Living 43 days......*	0	
,,	15	130	3·6	,, *43* ,, ...	0	This rat had 14 doses in 19 days, *vide* p. 6.
,,	16	155	1·5	,, *43* ,, ...	0	
,,	17	120	1·15	,, *43* ,, ...	0	
,,	18	225	3·75	Died on 31st day ...	2	No trypanosomes found *post mortem.*
,,	19	140	1·48	*Living 43 days......*	0	

,,	20	125	3·75	,, *43* ,, ……	3	Had 2 × 0·5 c.c. liq. hydrarg. perchlor. after first four doses of sodium antimonyl tartrate.
,,	21	150	1·5	,, *43* ,, ……	0	,, ,, ,,
,,	22	125	1·5	Died on 27th day…	1	Had 2 × 0·25 mg. succinimide of mercury after first four doses of sodium antimonyl tartrate.
,,	23	125	1·1	*Living 43 days*……	0	,, ,, ,,
,,	24	150	2·5	Died on 28th day…	1	Did not die of disease. No trypanosomes found.
,,	25	115	1·8	*Living 31 days*……	0	
,,	26	125	2·1	,, *31* ,, ……	0	
,,	27	125	2·1	,, *31* ,, ……	0	
,,	28	140	2·0	,, *31* ,, ……	0	
,,	29	190	1·5	,, *21* ,, ……	0	
,,	30	150	1·4	,, *21* ,, ……	0	
Surra ……	31	275	5·0	*Living 44 days*……	1	
,,	32	175	1·9	,, *44* ,, ……	0	
,,	33	175	1·5	,, *44* ,, ……	0	Had 2 × 0·5 c.c. liq. hydrarg. perchlor. after four doses of sodium antimonyl tartrate.
,,	34	175	1·5	,, *44* ,, ……	0	,, ,, ,,
,,	35	200	2·0	,, *44* ,, ……	0	Had 2 × 0·25 mg. succinimide of mercury after four doses of sodium antimonyl tartrate.
,,	36	150	1·5	,, *44* ,, ……	0	,, ,, ,,
,,	37	175	3·4	Died on 37th day…	0	Rat had eaten part of its fore-leg and tail, and was killed. No trypanosomes had been seen since the fourth day of the disease (and first of treatment). None were found *post mortem*, and two rats were inoculated with gland and marrow, with negative result.
,,	38	250	4·0	*Living 44 days*……	0	
,,	39	250	2·0	Died on 11th day…	0	Did not die of disease. No trypanosomes found after fourth day of disease (and first of treatment).

Sodium Arsenyl Tartrate or Sodium Tartrarsenite.—This compound was investigated by Henderson and Ewing* in 1895, who gave it the formula $AsONaC_4H_4O_6.2\frac{1}{2}H_2O$.

It was prepared for these experiments by dissolving one equivalent of arsenious oxide in two equivalents of acid sodium tartrate, filtering, evaporating to a small volume, and adding alcohol till crystallisation commenced; on cooling, the substance crystallised out.

This substance does not seem to be anything like so effective as the antimony compound. Of five rats which have been treated with it, four died between the 12th and 24th days, and three of these had a recurrence; one is still living on the 21st day, but we think that this is probably due to the sodium antimonyl tartrate which was given after a recurrence, in the same way as if from the beginning of the disease.

A mixture of equal parts of a 1 per cent. solution of sodium antimonyl tartrate and of sodium arsenyl tartrate has been tried on six rats. One died on the 14th day and the five others are still living on the 21st day without any recurrence.

Immunity.

With a view of ascertaining what amount of immunity, if any, had been conferred on an animal which we considered to be cured, a Nagana rat was taken which was inoculated on May 13, and had been afterwards successfully treated with atoxyl and succinimide of mercury, and in which no trypanosomes had been found since it had its first dose on May 16, when the trypanosomes were very plentiful in the blood. On October 7, the 147th day, the rat was re-inoculated from another Nagana rat, and on the 11th trypanosomes were present in numbers in the blood; a dose of sodium antimonyl tartrate was given and no trypanosomes have been seen since the 12th. This seems to point to the fact that no immunity is conferred.

DESCRIPTION OF PLATE.

The microphotographs were made from rough blood-preparations, with a low-power objective (Zeiss 8 mm.), in order to demonstrate the rapid disappearance of the trypanosomes from the blood after administration of sodium antimonyl tartrate.

Fig. 1 shows the blood of a Nagana rat, 4 days after inoculation, *before treatment.*

Fig. 2 shows the blood from the same rat *half an hour* after the injection of 0·35 c.c. of a 1 per cent. solution of sodium antimonyl tartrate.

Fig. 3 shows the blood from the same rat *one hour* after administration of the above dose.

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* Henderson and Ewing, 'Chem. Soc. Trans.,' 1895, vol. 67, p. 103.

Plimmer and Thomson. *Roy. Soc. Proc., B. vol.* 80, *Plate* 1.

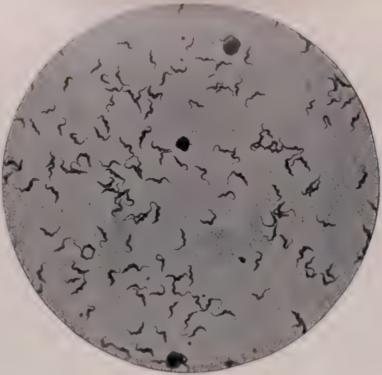

Fig. 1.

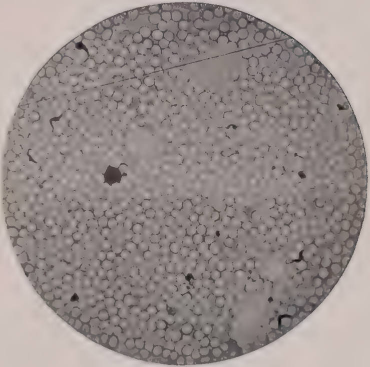

Fig. 2.

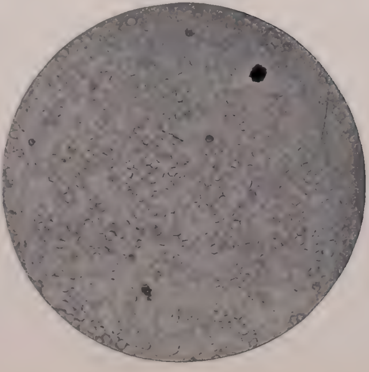

Fig. 3.

Note upon Sodium Antimonyl Tartrate.

By R. H. Aders Plimmer, D.Sc. Lond.

Sodium antimonyl tartrate was described in 1842 by Dumas and Piria,[*] who gave it the constitution $C_8H_8O_{10}NaO,Sb_2O_3H_2O$,[†] but did not state how they had prepared it. Clarke and Evans[‡] obtained a compound of the composition $3Na_2C_4H_4O_6 + 2Sb(OH)_3 + 3H_2O$, in 1883, by saturating tartaric acid with antimony trioxide and neutralising the solution with sodium carbonate. The first compound does not seem to have been prepared again since 1842.

Sodium antimonyl tartrate was prepared according to the methods usually given for preparing tartar emetic, by boiling a solution of acid sodium tartrate (13 grammes) with a little more than the calculated quantity (10 grammes) of antimony trioxide until the latter had almost completely passed into solution. On filtering and concentrating the solution to a small volume no crystallisation occurred, but on adding a little alcohol the whole became solid. This was then dissolved in about twice its volume of hot water, and alcohol was added until precipitation commenced, when, on cooling, the sodium antimonyl tartrate crystallised out. This compound at the ordinary temperature dries very slowly and has a moist appearance, but when dried *in vacuo* over sulphuric acid it becomes anhydrous and loses $2\frac{1}{2}$ molecules of water of crystallisation, resembling sodium tartrarsenite in this respect. The substance is very easily soluble in water and its solution reacts faintly acid to litmus.

0·7228 gramme air-dried substance lost *in vacuo* over sulphuric acid 0·0920 gramme H_2O = 12·73 per cent.
1·0538 „ „ „ „ 0·1338 gramme H_2O = 12·70 per cent.

Calculated for $C_4H_4O_7NaSb.2\frac{1}{2}H_2O$. $H_2O = 12{\cdot}8$ per cent.

I. 0·4058 gramme substance dried *in vacuo* over sulphuric acid gave 0·2332 gramme Sb_2S_3 and 0·0974 gramme Na_2SO_4.
II. 0·3780 gramme substance dried *in vacuo* over sulphuric acid gave 0·2072 gramme Sb_2S_3 and 0·0912 gramme Na_2SO_4.

Calculated for $C_4H_4O_7NaSb$. Sb = 39·09 per cent.; Na = 7·49 per cent.
Found :—I. Sb = 39·28 „ Na = 7·77 „
II. Sb = 39·14 „ Na = 7·81 „

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[*] Dumas and Piria, 'Liebig's Annalen,' 1842, vol. 44, p. 89.

[†] Old notation.

[‡] Clarke and Evans, 'Berichte,' 1883, vol. 16, p. 2385.

When dried at 105° C., the substance loses only two molecules of water of crystallisation :—

0·6120 gramme air-dried substance lost at 105° C. 0·0640 gramme H_2O = 10·46 per cent.

Calculated for $C_4H_4O_7NaSb.2H_2O$. H_2O = 10·49 per cent.

The remaining half molecule is subsequently lost *in vacuo* over sulphuric acid.

0·6120 gramme substance dried at 105° C. then lost *in vacuo* over sulphuric acid 0·0110 gramme H_2O. Total loss = 12·25 per cent.

On exposure to air, the two and a half molecules of water of crystallisation are again taken up, but the salt does not deliquesce.

0·5370 gramme substance dried *in vacuo* over sulphuric acid, on exposure to air increased in weight to 0·6066 gramme.

HARRISON AND SONS, Printers in Ordinary to His Majesty, St. Martin's Lane.

CLXXV.—*The Condensation of Acetaldehyde and its Relation to the Biochemical Synthesis of Fatty Acids.*

By Henry Stanley Raper.

The formation of fatty acids in animals, from carbohydrates, and the occurrence in natural fats, such as butter, of all the fatty acids, containing an even number of carbon atoms, from two to twenty, suggest that these fatty acids are produced by the condensation of some highly reactive substance containing two carbon atoms and formed in the decomposition of sugar.

It has been suggested by Nencki that in the butyric fermentation of lactic acid, acetaldehyde, carbon dioxide, and hydrogen are first formed and that two molecules of the acetaldehyde then unite to form butyric acid:

$$CH_3\cdot CH(OH)\cdot CO_2H = CH_3\cdot CHO + CO_2 + H_2.$$
$$2CH_3\cdot CHO = C_3H_7\cdot CO_2H.$$

Magnus Levy (*Englemann's Archiv*, 1902, 365) and Leathes (*Problems in Animal Metabolism*, London, 1906) have extended this suggestion to explain the formation of the higher fatty acids. Their conclusions receive support from the fact that *n*-hexoic acid has long been known to be a regular product of the butyric fermentation, and it has already been shown in a former communication (*Proc. Physiol. Soc.*, 1907, xxiv) that *n*-octoic acid is also produced to a small extent. According to the hypothesis referred to, therefore, in the formation of fatty acids from dextrose, lactic acid is the first product, and on the decomposition of this into acetaldehyde, carbon dioxide, and hydrogen, condensation of the aldehyde occurs, leading to the formation of the higher acids.

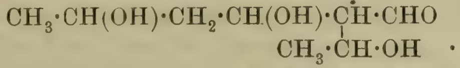
The chief difficulty in the acceptance of these views lies in the fact that all the previous work on the condensation of aliphatic aldehydes has shown that the products of condensation are aldehydes with branched, and not straight, chains (Lieben, *Monatsh.*, 1901, **22**, 289). This is due to the tendency of the aldehyde group of one molecule to condense with the α-carbon atom of the other molecule. Thus propaldehyde on condensation gives rise to β-hydroxy-α-methylvaleraldehyde and not to hexaldehyde:

$$2CH_3\cdot CH_2\cdot CHO = CH_3\cdot CH_2\cdot CH(OH)\cdot CH(CH_3)\cdot CHO.$$

But it must not be forgotten that Lieben's deductions have been confined to observations made on the condensation of aldehydes, such as acetaldehyde, propaldehyde, and valeraldehyde, which contain no hydroxyl groups; and, since when acetaldehyde condenses with itself the first product is necessarily β-hydroxybutyraldehyde (aldol), it is possible that the presence of the hydroxyl group in the β-position might influence the further course of the reaction, and conceivably lead to the production of the aldehyde with a straight chain.

The present paper deals with a study of this condensation, the first stage of which, namely, the formation of an aldehyde with eight carbon atoms from aldol, has been investigated. Previous observations on this subject are to be found in a paper by Wurtz (*Compt. rend.*, 1880, **91**, 1030), in which a substance, dialdane, $C_8H_{14}O_3$, is described as a product of the condensation of acetaldehyde under the influence of hydrochloric acid. The substance is regarded as possessing the constitution $CH_3\cdot CH(OH)\cdot CH_2\cdot CH{:}CH\cdot CH(OH)\cdot CH_2\cdot CHO$, but this formula is unsupported by evidence.

If the condensation of aldol followed the rule established by Lieben and his collaborators, then the first product of condensation should be $\beta\delta$-dihydroxy-α-ethanolhexaldehyde,



$$CH_3\cdot CH(OH)\cdot CH_2\cdot CH(OH)\cdot \underset{CH_3\cdot CH\cdot OH}{\underset{|}{CH}}\cdot CHO \cdot$$

By condensation under the influence of dilute potassium carbonate, aldol has been found to yield, not this substance, but a hydroxyoctaldehyde with a straight chain. Unfortunately, this aldehyde has never been obtained quite pure, and on this account a more complete examination of its structure has had to be abandoned. Analyses made with the substance purified as far as was possible indicate that on condensation a molecule of water is eliminated with the formation of an inner anhydride and not of an unsaturated compound as is generally the case. Under the action of traces of mineral acid, two molecules of the substance lose a molecule of water, forming an aldehyde, $C_{16}H_{26}O_5$.

That the carbon atoms are in a straight chain in the condensation product has been proved by oxidising it to the corresponding acid and then reducing this by means of hydriodic acid. In this way, *n*-octoic acid was obtained, and identified by means of its amide and barium salt. α-Ethylhexoic acid, which would have been obtained at this stage had the condensation taken place according to Lieben's rule, has been prepared for comparison.

EXPERIMENTAL.

The aldol used in the experiments was prepared by the condensation of acetaldehyde in presence of 5 per cent. potassium carbonate solution (Orndorff and Newbury, *Monatsh.*, 1892, **13**, 516).

Condensation of Aldol.—As condensing agent, a 5 per cent. solution of potassium carbonate was used. One hundred grams of freshly prepared aldol were mixed with 100 c.c. of distilled water and the solution cooled in ice. Five grams of solid potassium carbonate were then added in small portions, the solution being kept below 10° during the process. When the potassium carbonate had completely dissolved, the solution was kept at a temperature of 13—14° until a yellow oil had separated at the bottom of the vessel. The time occupied was about sixty hours. The solution was now diluted to 800 c.c. with water, rendered slightly acid with hydrochloric acid, and the yellow, tarry substance separated. The filtrate was extracted with 200 c.c. of ether in order to separate a little of the tarry matter remaining in solution from the condensation product, which is much less soluble. Barium carbonate was now added to the solution to neutralise the free acid, and the whole was distilled in steam for three hours to remove unchanged aldol. As the condensation product is also volatile to a small extent, a little is lost in this process. The solution in the distilling flask was now concentrated to a syrup by evaporating the water under reduced pressure, and extracted with ether. After drying the ethereal extract over anhydrous copper sulphate, the ether was removed by distillation and a pale yellow, very viscous liquid remained. The yield was 35 per cent. of the aldol taken.

The substance decomposed when distilled under the ordinary as well as under reduced pressure. It did not give crystalline derivatives by the usual methods applicable to aldehydes, although it gave the usual aldehyde reactions. On warming with a trace of calcium chloride or mineral acid, or on heating alone for some time, it gave rise to a new compound, which, unlike the parent substance, was less soluble in hot water than cold.

The molecular weight was determined by the boiling point method. For this purpose, the substance was heated to 50° under diminished pressure in a stream of dry carbon dioxide to remove the last traces of ether and moisture. The substance so obtained gave a slight opalescence on dissolving in water, which increased on warming:

0·7121 in 9·05 of alcohol raised the boiling point 0·47°. M.W. = 193.
0·7121 „ 13·70 „ „ „ 0·32°. M.W. = 187.
$C_8H_{16}O_4$ requires M.W. = 176. $C_8H_{14}O_3$ requires M.W. = 158.

On analysis:

0·1706 gave 0·3833 CO_2 and 0·1347 H_2O. C = 61·28; H = 8·77.
$C_8H_{14}O_3$ requires C = 60·76; H = 8·86 per cent.
$C_8H_{16}O_4$ „ C = 54·54; H = 9·09 „

These analytical results indicate that a molecule of water is eliminated in the process of condensation.

The high value obtained for the molecular weight is due to the presence of a substance derived from two molecules of the aldehyde, $C_8H_{14}O_3$, by the removal of water, This change, as stated above, takes place very readily on warming with a trace of calcium chloride or mineral acid, or merely by heating alone for some time. The new substance thus formed is very easily recognised by being much less soluble in hot water than cold; its cold aqueous solution immediately becomes milky on warming. On analysis:

0·2091 gave 0·4973 CO_2 and 0·1607 HO_2. C = 64·88; H = 8·54.
0·5250, in 7·37 of alcohol, gave an elevation of 0·28°. M.W. = 293.
0·5250 „ 11·74 „ „ „ 0·18°. M.W. = 286.
$C_{16}H_{26}O_5$ requires C = 64·43; H = 8·72 per cent. M.W. = 298.

It was found impossible to obtain a specimen of the condensation product quite free from this anhydride, the mere heating in a vacuum in order to dry the substance for analysis causing a certain amount of dehydration with consequent production of the anhydride. On this account, the analytical results can only be taken as approximately correct. When dissolved in acetic acid, the condensation product absorbed only a trace of bromine, so that it appears to be a saturated compound. It seems probable, therefore, that the water which is eliminated during condensation comes from two hydroxyl groups and thus gives rise to an inner anhydride.

Oxidation of the Condensation Product.

Freshly precipitated silver oxide in the presence of barium hydroxide was used as the oxidising agent. The silver oxide was obtained by precipitating silver nitrate with the requisite amount of barium hydroxide and washing until free from barium nitrate. It was not allowed to cake during the washing, as this materially affected the yield of acid.

Ten grams of the condensation product dissolved in 30 c.c. of water were added to 40 grams of freshly precipitated silver oxide suspended in about 400 c.c. of water. The mixture was cooled to 10°, and a solution of 10 grams of barium hydroxide in 200 c.c. of water added in portions of about 25 c.c. every five minutes. After each addition, the whole was well shaken and the temperature kept about 10°. When all the barium hydroxide had been added, the contents of the vessel were allowed to regain the room temperature. The shaking was continued at intervals of fifteen minutes for two hours. At the end of this time, the oxidation was usually complete. The silver and unchanged silver oxide were filtered off and well washed with distilled water, the filtrate and washings were mixed, and any excess of barium hydroxide precipitated by carbon dioxide. After filtering, the solution was distilled under reduced pressure until the residue became syrupy. This was dissolved in alcohol, filtered, if necessary, and the barium salt precipitated by the addition of ether. To remove any unchanged substance, the precipitate was re-dissolved in alcohol and again precipitated. The barium salt of the acid corresponding to the aldehyde, $C_8H_{14}O_3$, was thus obtained as a yellowish-white, amorphous, and very hygroscopic solid. It was dried at 100°:

0·2781 gave 0·1385 $BaSO_4$. Ba = 29·30.

$(C_8H_{13}O_4)_2Ba$ requires Ba = 28·34 per cent.

The free acid was obtained by decomposing the barium salt with the calculated amount of sulphuric acid. On filtering off the barium sulphate, boiling with a little animal charcoal, again filtering, and evaporating the filtrate under reduced pressure, the free acid was obtained as a very viscous liquid. The yield amounted to 70 per cent. The product did not crystallise even after standing for a considerable time in a vacuum over sulphuric acid. The lead, silver, calcium, and copper salts were prepared. The silver salt decomposed on drying. The lead, calcium, and copper salts were amorphous and very soluble, both in alcohol and water.

Reduction of the Acid, $C_8H_{14}O_4$, *with Hydriodic Acid.*

Ten grams of the free acid were boiled for five hours under a reflux condenser with 80 grams of hydriodic acid (b. p. 127°) and 4 grams of red phosphorus. After cooling, the liquid was diluted with twice its volume of water and extracted several times with ether. The ethereal extract was shaken with mercury to remove free iodine and the ether then removed by distillation. A thick brown oil was left behind, which still contained combined iodine. To remove this, dilute sulphuric acid was added and then zinc dust in small portions. After standing for twenty-four hours, the volatile acids were removed by distillation in steam. The distillate was neutralised with sodium hydroxide and evaporated to dryness on the water-bath. The yield of sodium salts amounted to 20—25 per cent. of the acid taken for the reduction.

5·5 Grams of the sodium salts were decomposed with sulphuric acid, and the fatty acids thus liberated were extracted with ether and dried over anhydrous sodium sulphate. The ether was removed on the water-bath and the residual fatty acid distilled. 0·85 Gram of acid passed over at 215—233°. Below 215°, a little butyric acid was obtained.* The fraction boiling at 215—233° was converted into the amide by Aschan's method (*Ber.*, 1898, **31**, 2348). After crystallising from light petroleum and then from water, the amide was obtained in glistening plates, which melted sharply at 105—106°, corresponding with the melting point of *n*-octoamide. When mixed with pure *n*-octoamide, the melting point was unchanged.

From another portion of the acid, the barium salt was prepared by neutralising with barium hydroxide. It was crystallised twice from water in order to remove barium butyrate, and was obtained in colourless plates :

0·0495 gave 0·0274 $BaSO_4$. Ba = 32·55.

$(C_8H_{15}O_2)_2Ba$ requires Ba = 32·39 per cent.

The acid obtained from the condensation product by oxidation and reduction is therefore octoic acid; hence the first stage in the condensation of aldol results in the production of a hydroxyaldehyde with the carbon atoms united in a straight chain.

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* This was due to the aldol not being completely removed from the condensation product by distillation in steam. In cases where the condensation product was not required for analysis, the steam distillation was shortened in order to lose as little as possible of the condensation product which is slightly volatile in steam.

The residue, which did not distil at 233°, was converted into the barium salt and analysed:

0·1312 gave 0·0727 $BaSO_4$. $Ba = 32·61$.

This was therefore barium octoate.

Synthesis of α-Ethylhexoic Acid.

This was carried out in the usual manner from ethylmalonic ester and butyl iodide. 1·7 Grams of sodium were dissolved in 20 c.c. of absolute alcohol and, after cooling, 14 grams of ethylmalonic ester added. 13·5 Grams of *n*-butyl iodide were now added, drop by drop, with frequent shaking. When all the butyl iodide had been run in, the mixture was heated on the water-bath for six hours, after which the alcohol was removed as completely as possible by distillation. The residue was dissolved in water, calcium chloride solution added, and the *ethylbutylmalonic ester* extracted with ether. After dehydrating the ethereal solution, the ether was removed on the water-bath and the ester distilled. Nine grams were obtained boiling at 235—245°. The ester was hydrolysed by boiling with the requisite amount of 30 per cent. aqueous potassium hydroxide. When hydrolysis was complete, the solution was extracted with ether to remove a little oily impurity, then acidified with dilute sulphuric acid, and the liberated acid extracted with ether. The ether was evaporated and the acid purified by crystallisation from water. It was obtained in colourless needles melting at 116°:

0·1035 gave 0·2191 CO_2 and 0·0769 H_2O. $C = 57·72$; $H = 8·26$.
$C_9H_{16}O_4$ requires $C = 57·44$; $H = 8·51$ per cent.

The ethylbutylmalonic acid was decomposed by heating to 165° for half an hour. The resulting α-ethylhexoic acid boiled at 225°. The acid was converted into the *amide* by Aschan's method (*loc. cit.*). It crystallised from water in long needles melting at 101—102°:

0·1454 gave ammonia which neutralised 10·30 c.c. $N/10$ H_2SO_4. $N = 9·7$.
$C_8H_{17}ON$ requires $N = 9·8$ per cent.

The barium salt of the acid has been previously described by Raupenstrauch (*Monatsh.*, 1887, 8, 115), who obtained the acid by the oxidation of the corresponding alcohol. Raupenstrauch states that the barium salt is amorphous. This was confirmed by preparing the barium salt of the synthetic acid which was also found to be amorphous.

α-Ethylhexoic acid is therefore easily distinguished from *n*-octoic acid by the melting point and crystalline form of its amide, and also by the fact that *n*-octoic acid gives a crystalline barium salt, whereas *a*-ethylhexoic acid does not.

In conclusion, I wish to express my thanks to Dr. Leathes for much kind help and advice during the course of the work.

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CULTIVATION OF THE TRYPANOSOME FOUND IN THE BLOOD OF THE GOLD-FISH.

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Plate III.

Since 1903 when McNeal and Novy first recorded that they had obtained cultures of *T. lewisi*, other trypanosomes found in the blood of mammals, more especially the pathogenic ones, *T. brucei* and *T. evansi*, have been successfully cultivated. Besides those parasitic in the blood of mammals, trypanosomes found in the blood of birds, of amphibians, and lastly those found in the stomachs of mosquitoes and other insects, have been grown on artificial media; but so far there has been no record of the successful cultivation of any species of fish trypanosome.

Brumpt (1906), after having described the evolution of fish trypanosomes, particularly those of the eel, in *Hemiclepsis*, and their behaviour in other species of leeches—*Callobdella punctata, Hirudo troctina, Piscicola geometra*—says that in the medium of McNeal and Novy he was unable to obtain any culture, but that in spite of the difference in the constitution of the medium as compared with that of the blood of the eel he observed some perfectly typical trypanosomes eight or ten days after inoculation. From his various experiments he concludes that the morphological changes which he observed in the trypanosomes were not due to any physical characters of the medium in which they developed, but were the result of a vital reaction of the parasites to a special chemical medium in a particular host.

Lebailly (1906) writes that, contrary to Brumpt's experience, he has found profound changes take place in fish trypanosomes "*in vitro*." The conditions of his experiments were not the same as those of Brumpt's, as Lebailly himself allows. In place of making use of the medium of

McNeal and Novy he simply kept films of blood containing trypanosomes between coverslip and slide by sealing round the edges of the coverslip with paraffin wax. By the end of eight or ten days almost all the trypanosomes in the film had changed into "small granular spherical masses each with flagellum feebly moving."

During the months of May, June and July of 1906, acting on the advice of Dr Dean and helped by him and by Dr Ledingham, I succeeded in obtaining cultures in blood-agar tubes of a trypanosome found in the blood of gold-fishes taken from a pond at Queensbury Lodge, Elstree. The pond is about twenty yards long by about fifteen yards broad. It has a muddy bottom, and contains many weeds and decaying leaves. There is a small inlet and a small outlet so that the water in the pond is slowly changing. In this pond the gold-fishes have been allowed to multiply and to feed naturally for many years. Every fish whose blood I examined harboured trypanosomes. As I examined a great many of all sizes from 1 cm. up to 20 cm. long (the caudal fin which was often damaged being omitted in these measurements), I believe that every fish in the pond from 1 cm. in length upwards is infected. On the other hand I was constantly on the outlook for leeches and although on one occasion (28th May) I had the pond dragged, and also carefully examined with the aid of a hand-lens the gills and the surface of the body between the scales of 178 fishes, I never succeeded in finding a leech. These two facts taken in conjunction —the failure to find leeches during the months of May, June and July, and the constant presence of trypanosomes in every fish examined from 1 cm. in length upwards—suggest that in addition to the known mode of transmission by the leech, there may possibly be some other way or ways by which fish may become infected with trypanosomes.

Description of the trypanosome as it is found in the blood of the gold-fish.

This gold-fish is a species of carp, and the trypanosome found in it (Plate III, Figs. 1 and 2) is, so far as can be judged from its morphology, identical with *T. danilewskyi*, as described by Laveran and Mesnil (1904). As seen in films fixed by methyl alcohol and stained by Giemsa, the body measures 35—45μ long and about 3μ broad; the free flagellum is 15—17μ long; the posterior end of the body is pointed; the kinetonucleus[1], situated about 2·5μ from the posterior extremity, is prominent, in dried specimens frequently seen projecting from one side of the body like a short horn: the undulating membrane is large and well folded: the tropho-nucleus is elongated, being about 4 μ long by about 2 μ broad, and lies somewhat nearer to the anterior than to the posterior end of the body: in addition to the tropho- and kineto-nuclei, the body protoplasm contains chromatic granules varying in number and in size.

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[1] The nomenclature here adopted is that suggested by Woodcock (1906). The smaller body is nuclear in nature, but the term micro-nucleus has already been appropriated for

Cultivation.

The culture medium found successful was that recommended by McNeal and Novy for the culture of *T. brucei*, slightly modified by Nocht and Mayer, viz.,

I. Extract of 125 grammes of ox flesh in 1000 grammes of water.

Agar	25 grammes.
Peptone	20 grammes.
Chloride of Sodium	5 grammes.
Normal carbonate of Soda solution	10 c.c.

II. Defibrinated rabbit's blood.

One volume of I while still fluid at a temperature of 55° to 60° C. is mixed with two volumes of II in test tubes and the mixture allowed to set in the tube kept sloped in the ordinary way. After the medium has set the tubes are placed upright, capped, and incubated for 24 hours to ensure that the contents are sterile.

Tubes prepared as above were inoculated with blood drawn aseptically from the heart of an infected fish immediately after it had been killed, and were kept in a cool chamber at a temperature of about 15° C. With this medium and under these conditions the proportion of successes was one in three.

The chief difficulties encountered were the technical ones of obtaining blood from the infected fish in an aseptic condition and in transferring this blood to the culture tubes. The heart in these small fishes is very friable and the blood is very coagulable. Capillary pipettes were used to withdraw the blood from the heart. In introducing the point of the pipette great care had to be taken to avoid tearing through the heart wall at the point of fixation. The next difficulty arose from the coagulability of the blood. A little citrate of soda solution (1 %) was

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the generative nucleus of the Ciliata. Several observers have drawn attention to the fact that the flagellum arises from a small basal granule distinct and separate from the smaller or kinetic nucleus. Professor Minchin referring to these points during the course of his lectures at the Lister Institute defined the blepharoplast as an achromatic body, or centrosome in relation to the flagellum; and was of opinion that the term blepharoplast so often used for the kinetic nucleus should be confined to this basal granule. Whenever the term "Blepharoplast" is used in this paper, it refers strictly to this basal granule.

first drawn into the tube; but even then no time had to be lost in transferring the drawn blood to the culture tube.

Results with rabbit-blood-agar medium.

In tubes that gave successful cultures, developmental forms were first seen on the 6th and 7th days after inoculation, though very probably developmental changes had begun earlier than this. Both living and stained preparations were examined at different stages of the culture. Of living preparations it will suffice to say that small free actively swimming forms were seen at all stages of the culture. These could pass quickly across the field of a 1/12th objective. Large dividing forms, often with several flagella, though they showed very active flagellar movements, had very limited movements of translation. Drawings of stained preparations from a successful culture at different periods after inoculation are reproduced on Plate III, and from these morphological changes can more easily be studied.

Description of stained preparations.

The slides from which these drawings were made were prepared in the following way: a drop taken from the culture tube was spread as a film on a slide, fixed in methyl alcohol, treated with fresh blood serum in the way recommended by Leishman (1904) for sections of tissues to be stained by Romanowsky stains, stained by Giemsa (4 drops to 1 c.c. distilled water for 5 hours), then washed, dried and mounted.

1. *Seventh day of the culture.* Plate III, Figs. 3 to 11, are from a film prepared from culture on the 7th day after inoculation. Fig. 3 represents one of two apparently unaltered forms found in this film. Figs. 4 to 11 have been selected to show how the various cultural forms have been derived from the original trypanosome form as found in the blood of the fish. The initial change seems to consist in a concentration of the endoplasm and a bringing together of endoplasmic structures. The posterior two-thirds of the animal becomes greatly altered in shape and in arrangement of parts. It becomes short and swollen, while the anterior third remains much as it was, so that the whole animal assumes a somewhat tadpole-like appearance as seen in Fig. 4. The kineto-nucleus and the tropho-nucleus, in place of being separated from each other by nearly half the length of the animal, now lie side by side; and the undulating membrane is correspondingly shortened. The tropho-nucleus has become swollen and loose in

structure and its chromatin broken up into chromidia. At this stage apparently division can take place, and Fig. 5 shows division almost completed. The thick swollen end at this stage stains by Giemsa a deep purple. The anterior third unaltered in external form takes no part in the division. The product of division—resembling *Crithidia*—as seen still attached in Fig. 5, and free in Fig. 6, has apparently the power of multiplying freely. This is illustrated by Figs. 7, 8 and 9. Figs. 8 and 9 are probably agglutinations, but the point now is that in this *Crithidia*-like form free multiplication has taken place. Fig. 10 shows a form where the body has become elongated, but where the kineto- and tropho-nuclei are still close together. In Fig. 11 the kineto-nucleus has come to lie at a considerable distance behind the tropho-nucleus. As will be seen below, trypanosome-like forms (with body elongated and kineto-nucleus well behind the tropho-nucleus) become more numerous at later stages of the culture.

2. *Twenty-first day of the culture.* Figs. 12 to 15 inclusive are from a film prepared from a drop taken on the 21st day of the culture. The *Crithidia*-like form (Figs. 12 and 14) is still in evidence. Along with this are other forms with well developed undulating membrane (Fig. 15), and others where the kineto-nucleus is anterior to the tropho-nucleus and where division is going on (Fig. 13).

3. *The twenty-eighth day of the culture.* Plate III, Figs. 16 to 26, are from a film taken on the 28th day of the culture. The preponderance of bulky forms, the signs of considerable nuclear activity, and the presence of so many coarse granules in the body protoplasm are here very striking. The granular condition of the protoplasm may be due to some influence of medium (most probably the result of some accidental change that had taken place in it about this time) and may possibly be analogous to the granular condition of mast cells. Mast cells, as is known, are found in the mesentery more abundantly than in other parts of the body; and in it in greater numbers during digestion or after a meal. Forms with the kineto-nucleus close by the side of the tropho-nucleus—an arrangement seemingly favourable to free multiplication—are still found (Figs. 17 to 20 and Fig. 23). Fig. 16 shows multiple fission. Other forms more trypanosome-like than any hitherto seen in the culture now appear (Figs. 21 and 24). In Figs. 18, 23 and 25 the tropho-nucleus has divided before the kineto-nucleus, while in Figs. 21 and 24 the reverse is the case. In Fig. 21 it is difficult to see how the smaller of the dividing portions was to get its tropho-nucleus; but I have observed an exactly similar condition in a blood film from a rat that had

been inoculated with *T. lewisi*, so that such apparently anomalous forms are not confined to culture tubes. In Fig. 24 the true blepharoplasts or basal granules, from which the flagella arise distinct from the kineto-nuclei, are clearly seen.

4. *Forty-third day of the culture.* Figs. 27 to 35 are from a film taken on the 43rd day of the culture. In all, the body protoplasm is free from granules. It would thus appear that the influence or influences, to which was due the granular condition seen in forms from the 28th day of the culture, were of a temporary nature. Forms that are *Crithidia*-like by the relative position of kineto- and tropho-nuclei are here also seen; and multiplication by division is still actively going on. In Fig. 32 the kineto-nucleus is getting behind the tropho-nucleus; while in Figs. 33, 34 and 35 trypanosome-like forms resembling mammalian trypanosomes more closely than adult fish trypanosomes, are represented. These divide by unequal longitudinal division, and Fig. 33 shows division begun with the division of the blepharoplast. This is often seen to be the case in mammalian types. Division of the kineto-nucleus, whether it takes place before or after division of the tropho-nucleus, is probably always preceded by division of the true blepharoplast or basal granule, and commencing formation of a new flagellum. These trypanosome-like forms were found to be more numerous at this period than at earlier stages in the culture.

How long the culture lived after this I do not know. When I returned from a holiday seven weeks later it had died out. Sub-cultures that I inoculated just before leaving also showed no sign of life on my return.

Summary and Conclusions.

1. The trypanosome of the gold-fish has been successfully cultivated on the medium of McNeal and Novy.

2. Preparatory to division in culture, the original trypanosome as found in the blood of the fish assumes a somewhat tadpole-like appearance, the endoplasm and its contained structures being collected together in the swollen posterior end. The kineto-nucleus now lies close to, and alongside of, the tropho-nucleus, and the latter has become swollen and loose in structure with its chromatin broken up into chromidia. The anterior third or more of the trypanosome undergoes little or no change in form and does not take part in division. It is thus easily seen how the product of the preliminary division comes to have a *Crithidia*-like appearance.

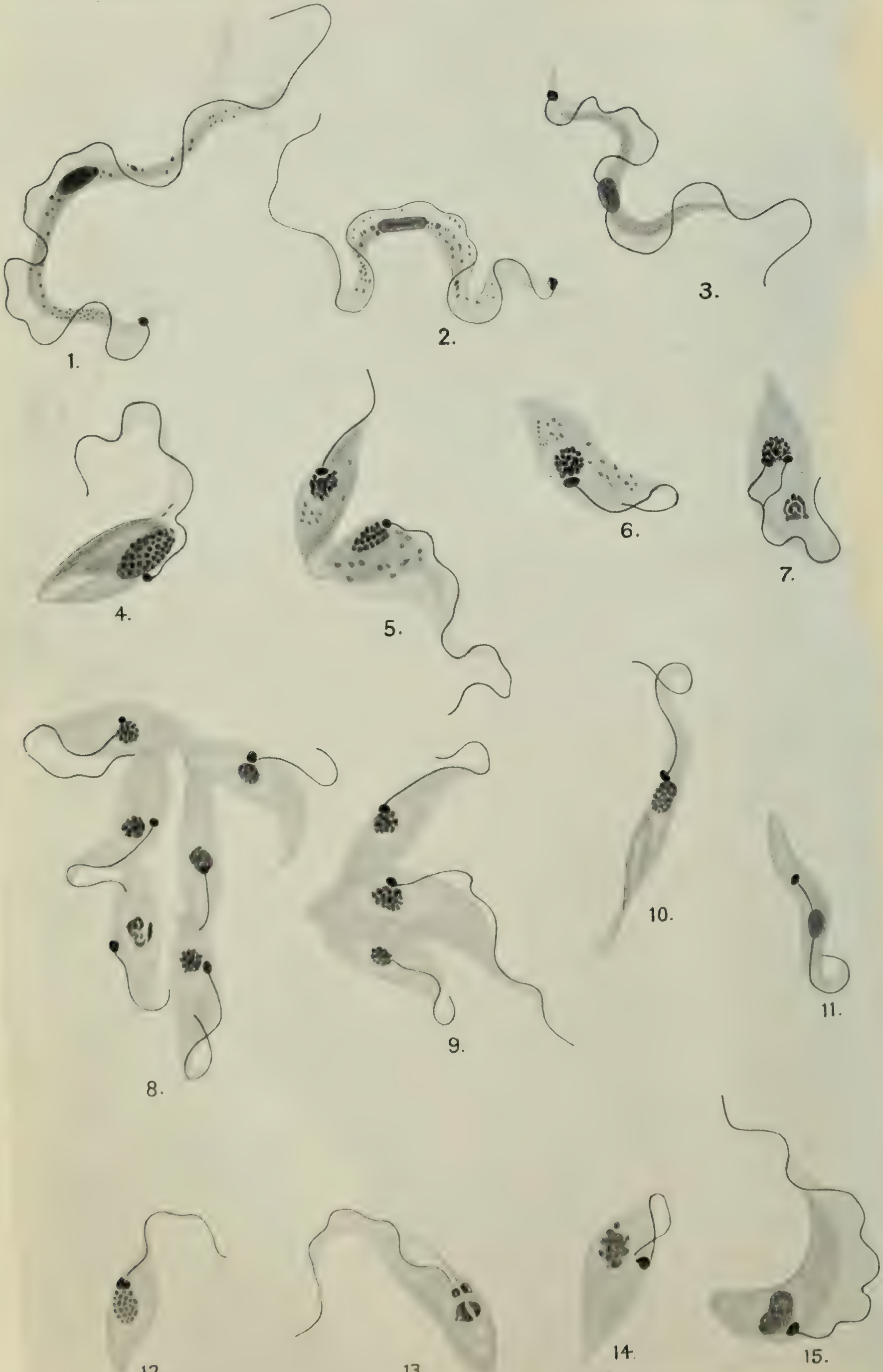
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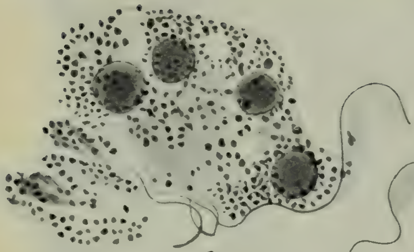
PLATE III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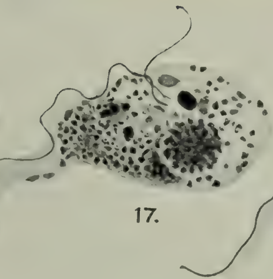
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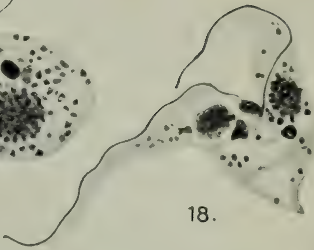
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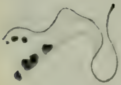
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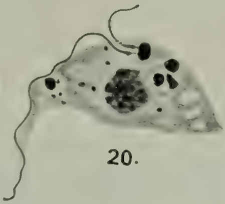
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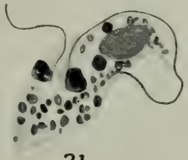
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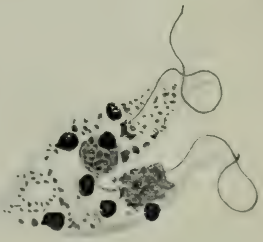
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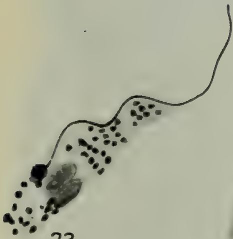
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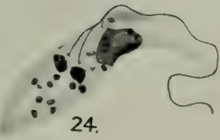
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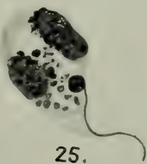
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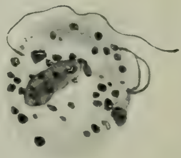
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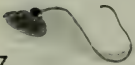
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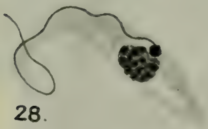
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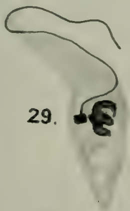
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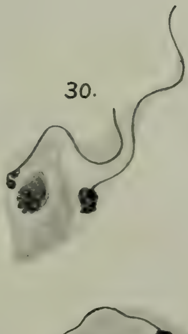
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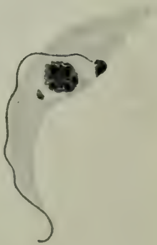
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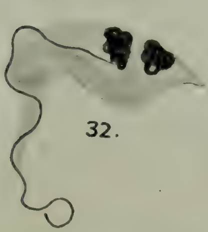
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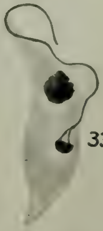
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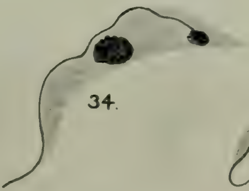
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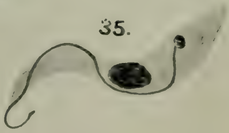
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3. The product of this preliminary division—*Crithidia*-like by the relative position of kineto- and tropho-nuclei—is capable of freely multiplying.

According to Brumpt it is in the *Crithidia*-like form that free multiplication of the eel trypanosome first takes place in the stomach of *Hemiclepsis*. It may well be that in this case also the *Crithidia*-like form is arrived at by steps such as are here figured and described.

4. *Crithidia*-like forms are found at all stages of the culture and along with them at various stages other forms where the body is elongated and the kineto-nucleus still close to the tropho-nucleus, and yet other forms where the kineto-nucleus lies at varying distances behind the tropho-nucleus until true trypanosome-like forms are reached.

5. These trypanosome-like forms (resembling mammalian trypanosomes rather than those of the fish from which they are derived) are most numerous in the later stages of the culture.

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DESCRIPTION OF PLATE III.

Figs. 1 and 2. Trypanosomes from film of blood of gold-fish.

Figs. 3—11. Forms found in film prepared from culture on the 7th day after inoculation. Fig. 3, unaltered form. Fig. 4, form showing the change that takes place preparatory to division. Fig. 5, primary division almost completed. The anterior part of the trypanosome which retains its original form takes no part in division, and the product of division is *Crithidia*-like in appearance. Figs. 6—9, *Crithidia*-like forms that multiply freely. Fig. 10, form with body elongated but with kineto-nucleus still close to (here somewhat anterior to) the tropho-nucleus. Fig. 11, form where the kineto-nucleus has come to lie posterior to the tropho-nucleus.

Figs. 12—15. Forms from a film prepared from cultures on the 21st day after inoculation. Figs. 12 and 14, *Crithidia*-like forms. Fig. 13, dividing form with kineto-nucleus anterior to the tropho-nucleus. Fig. 15, form with well-developed undulating membrane.

Figs. 16—20. Forms from film prepared from culture on the 28th day after inoculation. The bulky form of some, the number of dividing forms and the coarse granular appearance of all are conspicuous. In Figs. 18, 23 and 25 the tropho-nucleus has divided before the kineto-nucleus : in Figs. 21 and 24 the reverse is the case : while in Figs. 17 and 20 the true blepharoplasts are the first to divide. Fig. 24 shows clearly the origin of the flagella from small basal granules, or true blepharoplasts, distinct from the kineto-nuclei.

Figs. 25—35. Forms from film prepared from culture on the 43rd day after inoculation. Note the absence of granules in the protoplasm, the *Crithidia*-like forms, dividing forms, and trypanosome-like forms—the latter being more numerous than at earlier stages of the culture.

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